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Suppression of Sclerostin Alleviates Radiation-Induced Bone Loss by Protecting Bone-Forming Cells and Their Progenitors Through Distinct Mechanisms

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Abstract

Focal radiotherapy is frequently associated with skeletal damage within the radiation field. Our previous in vitro study showed that activation of Wnt/ β -catenin pathway can overcome radiation-induced DNA damage and apoptosis of osteoblastic cells. Neutralization of circulating sclerostin with a monoclonal antibody (Scl-Ab) is an innovative approach for treating osteoporosis by enhancing Wnt/ β -catenin signaling in bone. Together with the fact that focal radiation increases sclerostin amount in bone, we sought to determine whether weekly treatment with Scl-Ab would prevent focal radiotherapy-induced osteoporosis in mice. Micro-CT and histomorphometric analyses demonstrated that Scl-Ab blocked trabecular bone structural deterioration after radiation by partially preserving osteoblast number and activity. Consistently, trabecular bone in sclerostin null mice was resistant to radiation via the same mechanism. Scl-Ab accelerated DNA repair in osteoblasts after radiation by reducing the number of γ -H2AX foci, a DNA double-strand break marker, and increasing the amount of Ku70, a DNA repair protein, thus protecting osteoblasts from radiation-induced apoptosis. In osteocytes, apart from using similar DNA repair mechanism

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to rescue osteocyte apoptosis, Scl-Ab restored the osteocyte canaliculi structure that was otherwise damaged by radiation. Using a lineage tracing approach that labels all mesenchymal lineage cells in the endosteal bone marrow, we demonstrated that radiation damage to mesenchymal progenitors mainly involves shifting their fate to adipocytes and arresting their proliferation ability but not inducing apoptosis, which are different mechanisms from radiation damage to mature bone forming cells. Scl-Ab treatment partially blocked the lineage shift but had no effect on the loss of proliferation potential. Taken together, our studies provide proof-of-principle evidence for a novel use of Scl-Ab as a therapeutic treatment for radiation-induced osteoporosis and establish molecular and cellular mechanisms that support such treatment.

Keywords

RADIOTHERAPY; SCL-AB; OSTEOBLASTS; MESENCHYMAL PROGENITORS; DNA REPAIR

Introduction

Radiotherapy destroys cancer cells using high-energy ionizing radiation to damage their DNA and induce cell death.⁽¹⁾ The efficacy of radiotherapy, either as a monotherapy or in combination with chemotherapy, surgery, and targeted medicines, accounts for its use in more than 50% of cancer patients, such that approximately 2 million patients per year in the US receive radiotherapy at some stage during their illness.⁽²⁾ The aim of radiotherapy is to target its destructive effects on tumor cells while limiting its damage to nearby healthy tissues. This has been improved greatly by computer-assisted treatment planning and innovations in technologies, such as proton and intensity-modified radiotherapy.⁽³⁾ However, loss of bone density and an elevated risk of fracture remain problematic late effects of radiotherapy.⁽⁴⁾ With the ever-increasing survival rate and longevity of cancer patients, understanding the mechanism of radiation-induced bone loss and identifying preventive or curative treatments are of greater clinical significance than ever before.

Whereas treatment of blood cancers, such as leukemia and lymphoma, involves total body irradiation with moderate doses of radiation, treatment of localized solid tumors, such as colon/rectum, breast, lung, prostate, cervix, and brain cancers, uses higher doses but more focal radiotherapy. Most animal studies analyzing radiation damage to bone either irradiate the entire animal or a large portion of the body, for example, a hindlimb, using lead shielding to cover the rest of the body. Similar to human subjects, irradiated mice experienced a marked decrease in trabecular bone mass starting from 2 weeks and persisting over 2 to 3 months^(5–11) that resulted from transiently elevated osteoclast activity at early stage after radiation^(8,9,11,12) and overall suppressed osteoblast activity.^(5,7,11) Antiresorptive agents, such as risedronate and zoledronic acid, were effective in blocking this type of bone loss.^(11,13) However, because of its extremely low dosage and extensive systemic effects, this type of radiation model might not truly mimic focal radiation commonly used to treat solid tumors in the clinic.

The recently available Small Animal Radiation Research Platform (SARRP) provides a new opportunity to study the mechanisms of focal radiotherapy-induced osteoporosis in rodents.

This irradiator is capable of delivering conformal and image-guided radiation with sub-millimeter accuracy, replicating the effects of focal radiation therapy in cancer patients.⁽¹⁴⁾ Using this approach, we recently demonstrated that the major mechanism for focal radiation damage to bone is the suppression of bone formation through apoptosis of osteoblasts and osteocytes, rather than enhancement of bone resorption.⁽¹⁵⁾ Therefore, injections of teriparatide (recombinant human parathyroid hormone [rhPTH] 1-34), which is an FDA-approved anabolic drug for postmenopausal osteoporosis, is highly protective of trabecular bone after focal radiation in young⁽¹⁶⁾ and adult rats.⁽¹⁵⁾ Further mechanistic investigation using in vitro and ex vivo cultures revealed that rhPTH1-34 blocks radiation-induced osteoblast apoptosis by accelerating the repair of DNA double-strand breaks (DSBs) via non-homologous end-joining (NHEJ) pathway.⁽¹⁷⁾ Interestingly, this action is mediated by β -catenin signaling and thus activation of the canonical Wnt pathway by treating osteoblastic cells with Wnt3a has similar effects as PTH in regulating DSB repair machinery and promoting survival after radiation.⁽¹⁷⁾

Sclerostin is a Wnt antagonist that binds to the Wnt coreceptor proteins LRP4/5/6^(18–20) and its expression in adults is largely restricted to osteocytes.^(21,22) Engineered knockout of sclerostin in mice and naturally occurring loss-of-function mutations in humans with the autosomal recessive disorders (sclerosteosis and van Buchem disease) are characterized by a high bone mass phenotype.⁽²³⁾ Therefore, targeting this protein leads to specific activation of the canonical Wnt signaling in bone. A monoclonal antibody against sclerostin (Scl-Ab) has been developed as a bone anabolic drug for postmenopausal osteoporosis.⁽²⁴⁾ In this study, we first examined whether Scl-Ab executes radioprotective actions on bone using a mouse focal radiation model. Mature bone-forming cells, osteoblasts, are short-lived cells with a half-life of about 2 to 4 weeks and, therefore, they need constant replenishment from bone marrow mesenchymal stem cells (MSCs) and their immediate descendants, osteoprogenitors. In addition to osteolineage cells, bone marrow MSCs also constitute bone marrow adipocytes, and marrow adiposity is an early detrimental event after radiation. Using a unique lineage tracing approach that allows us to visualize all these mesenchymal lineage cells inside mouse long bones, we then investigated the distinct mechanisms of radiation damage and Scl-Ab rescue effects on progenitors and osteo-differentiated cells in vivo.

Materials and Methods

Animal study design

All animal studies described in this article were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Pennsylvania. Specific pathogen-free male *C57BL/6* mice (8–10 weeks) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Age- and sex-matched *sclerostin* (*Sost*) *knockout* (*KO*) mice were kindly provided by Novartis (Basel, Switzerland). *Col2-Cre Rosa-Tomato* (*Col2/Tomato*) mice were generated by breeding *Col2-Cre* mice and *Rosa-Tomato* mice obtained from Jackson Laboratory. In accordance with the standards for animal housing, mice were group housed at 23°C to 25°C with a 12-hour light/dark cycle and allowed free access to water and standard laboratory pellets. All animals were irradiated at the distal metaphyseal region of right femurs by SARRP (Xstrahl, Suwanee, GA, USA) at a clinically relevant dose

of 8 Gy twice, on days 1 and 3 as described previously.⁽¹⁵⁾ The radiation was delivered in a 5×5 mm square collimated field centered at the metaphysis about 1 mm below the growth plate at a rate of 1.65 Gy/min with the aid of built-in μ CT and X-ray. For Scl-Ab treatment experiments, mice were then divided into two groups with similar body weight at the outset of the study, receiving either vehicle (isotonic vehicle buffer, supplied from Novartis) or Scl-Ab (100 mg/kg/week, supplied from Novartis) weekly subcutaneous injections from day 1. The left femurs served as non-radiated paired controls because our previous studies demonstrate that focal SARRP radiation has no bone-damaging effects on contralateral legs.⁽¹⁵⁾ Serum was collected at time of death to determine osteocalcin (Mouse Osteocalcin Enzyme Immunoassay Kit, Alfa Aesar, Ward Hill, MA, USA) and CTX-I (RatLaps EIA, Immunodiagnostic Systems Inc., Gaithersburg, MD, USA) levels. Both focal radiation and Scl-Ab injections did not affect mouse body weight and cause any noticeable gross morphological or behavioral changes in mice.

Micro-computed tomography (μ CT) analysis

Four weeks after radiation, both femurs ($n = 7/\text{group}$) were harvested for μ CT analyses (microCT 35, Scanco Medical AG, Brüttisellen, Switzerland). Briefly, the distal end of the femur corresponding to a 0 to 4.1 mm region above the growth plate was scanned at 6 μm isotropic voxel size to acquire a total of 686 μ CT slices per scan. All images were first smoothed by a Gaussian filter (sigma = 1.2, support = 2.0) and then thresholded corresponding to 30% of the maximum available range of image grayscale values. The images of the secondary spongiosa regions 0.6 to 1.8 mm above the highest point of the growth plate were contoured for trabecular bone analysis. Geometric trabecular volumetric bone mineral density (vBMD), bone volume fraction (BV/TV), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), trabecular number (Tb.N), and structure model index (SMI) were calculated by 3D standard microstructural analysis.⁽²⁵⁾ Based on thresholded whole bone images, microstructural finite element (μ FE) models were generated by converting each bone voxel to an 8-node brick element. Bone tissue was modeled as an isotropic, linear elastic material with a Young's modulus of 15 GPa and a Poisson's ratio of 0.3. A uniaxial compression was applied along the axial direction of the model and the model was subjected to a linear elastic analysis to determine bone stiffness.

Static and dynamic histomorphometry

Mice were injected subcutaneously with calcein (15 mg/kg, Sigma-Aldrich, St. Louis, MO, USA) and xylenol orange (90 mg/kg, Sigma-Aldrich) at 9 and 2 days, respectively, before necropsy for dynamic measurements. After μ CT scans, femurs were processed for methyl methacrylate embedding. Using a Polycut-S motorized microtome, longitudinal sections were cut at 5 μm thickness followed by Goldner's trichrome staining for static analysis and at 8 μm thickness for dynamic measurements ($n = 5/\text{group}$). All images were quantified by Bioquant Osteo Software (Bioquant Image Analysis, Nashville, TN, USA). The primary indices include the total tissue area (TV), trabecular bone perimeter (BS), trabecular bone area (BV), osteoblast number (Ob.N), osteoclast surface (Oc.S), single- and double-labeled surface, interlabel width, and adipocyte number (Ad.N). Mineralizing surface (MS) and surface-referent bone formation rate (BFR/BS, $\mu\text{m}^3/\mu\text{m}^2/\text{d}$) were calculated as described by Dempster and colleagues.⁽²⁶⁾

Histology

Bilateral femurs ($n = 3/\text{group}/\text{time point}$) were harvested at various time points after radiation for histological analysis. They were fixed in 4% paraformaldehyde overnight, decalcified in 15% EDTA for 21 days, and processed for either paraffin (H&E staining) or frozen (immunohistochemistry) sections. For sclerostin staining, slides were first incubated with goat anti-sclerostin primary antibody (R&D Systems, Minneapolis, MN, USA; AF1589) at 4°C overnight, followed by binding with biotinylated secondary antibodies and DAB color development as well as methyl green counterstaining. The images were captured, inverted into RGB formats, and separated into different color stacks. Trabecular osteocytes, total around 250 to 300 per section, within the metaphyseal region at 1 to 2.5 mm above the growth plate were individually contoured. The intensity of sclerostin staining in each osteocyte was then quantified and normalized against the neighboring background area with a similar shape. The TUNEL assay was carried out according to the manufacturer's instructions (ApopTag Fluorescein In Situ Detection Kit, EMD Millipore, Billerica, MA, USA). To trace mesenchymal lineage cells, frozen sections from *Col2/ Tomato* mice were incubated with rabbit anti- γ -H2AX (Cell Signaling Technology, Danvers, MA, USA; 9718), mouse anti-Ku70 (Abcam, Cambridge, MA, USA; ab3108), rabbit anti-perilipin (Cell Signaling Technology, 9349), or rat anti-endomucin (Santa Cruz Biotechnology, Dallas, TX, USA; sc-65495) at 4°C overnight, followed by Alexa Fluor conjugated secondary antibodies. Sections were scanned by a Nikon Eclipse 90i fluorescence microscope. γ -H2AX foci were quantified in all Tomato⁺ osteoblasts and osteocytes within the metaphyseal region located at 1 to 2.5 mm above the growth plate using ImageJ software. Tomato⁺ bone marrow progenitors, Tomato⁺perilipin⁺ adipocytes, and Tomato⁺ pericytes on vessel surface, total around 350 to 450 Tomato⁺ bone marrow cells per section, were counted in the same region. To visualize osteocytic processes, cryosections were stained with DyLight 488-Phalloidin (Cell Signaling Technology, 12935). The length of canaliculi was measured on all trabecular osteocytes, total around 250 to 300 per section, using Bioquant Osteo Software (Bioquant Image Analysis, Nashville, TN, USA).

Analysis of bone marrow endosteal mesenchymal progenitors

Endosteal bone marrow cells were harvested as previously described.⁽²⁷⁾ Briefly, the outer surface of the femur was scraped several times followed by protease digestion (2 mg/mL collagenase A and 2.5 mg/mL trypsin in PBS) for 20 minutes to remove the periosteum and periosteal progenitors. Then, the radiated fragment (0 to 5 mm above the growth plate) and its contralateral fragment were longitudinally cut into two halves and digested by proteases again to collect endosteal bone marrow. These cells were either seeded for CFU-F assay (1×10^6 cells/T25 flask) or cultured for endosteal mesenchymal progenitors (1×10^6 cells/35 mm dish). When they reached confluency, mesenchymal progenitor cultures were then incubated with either osteogenic medium (α MEM with 10% FBS, 10 nM dexamethasone, 10 mM β -glycerophosphate, and 50 μ g/mL ascorbic acid) or adipogenic medium (α MEM with 10% FBS, 0.5 mM isobutylmethylxanthine, 10 mM indomethacin, 1 μ M dexamethasone, and 10 μ g/mL insulin) for differentiation. For radiation experiment, 70% confluent mesenchymal progenitor cells were irradiated (8 Gy) followed by apoptosis analysis via ethidium bromide (EB) and acridine orange (AO) (Sigma-Aldrich) staining at 2 days post-irradiation as

described previously.⁽²⁸⁾ Additional progenitor cells were trypsinized and reseeded after radiation for CFU-F assay.

Statistics

Data are expressed as means $\pm$ standard error (SEM) and analyzed by paired Student's *t* test for comparison of irradiated and non-irradiated contralateral femurs and by unpaired Student's *t* test for comparison of vehicle and Scl-Ab-treated samples followed by Bonferroni adjustment for multiple comparisons using Prism 5 software (GraphPad Software, San Diego, CA, USA). Values of $p < 0.05$ were considered statistically significant.

Results

Focal radiation increases sclerostin amount in trabecular bone

We first analyzed whether focal radiation itself alters sclerostin amount in bone. At 2 weeks after radiation, immunohistochemistry (IHC) clearly showed that sclerostin amount was significantly increased in trabecular osteocytes within the irradiated area compared with those located in non-irradiated area (Fig. 1A, B). Scl-Ab injections did not change sclerostin amount regardless of radiation. These results provided additional evidence supporting Scl-Ab as a potential therapy for radiation-induced bone damage. Scl-Ab injections at 2 weeks increased total β -catenin amount by 3.5-fold in bone surface osteoblasts, and similar to a recent report,⁽²⁹⁾ the mRNA level of *DKK1*, a direct transcriptional target of β -catenin, in metaphyseal bone (Supplemental Fig. S1), implying that this treatment does activate Wnt/ β -catenin signaling pathway.

Inhibition of sclerostin blocks SARRP radiation-induced trabecular bone damage in mice

In line with our previous reports that focal radiation decreases trabecular bone mass and causes microarchitectural damage in young and adult rats,^(15,16) SARRP radiation of the right distal femoral region of *WT* mice treated with vehicle led to robust trabecular bone structural deterioration (Fig. 2A) with marked decreases in volumetric bone mineral density (vBMD, 30%), bone volume fraction (BV/TV, 31%), and trabecular number (Tb.N, 13%) accompanied by increases in trabecular separation (Tb.Sp, 19%) and structure model index (SMI, 26%) after 4 weeks (Fig. 2B). Furthermore, μ FE analysis revealed a 56% reduction in trabecular bone stiffness in irradiated bones compared with non-irradiated controls (Fig. 2C), consistent with the notion that radiation-induced trabecular bone loss leads to impaired mechanical strength. By contrast, 4 weeks of Scl-Ab injections, starting immediately after the initial SARRP radiation, not only blocked any structural deterioration but also strikingly increased bone mass and improved bone quality in the radiated area to the same levels as in non-radiated area with Scl-Ab treatment (Fig. 2). Compared with vehicle-treated irradiated bones, Scl-Ab-treated irradiated trabecular bones had approximate 2.5-, 2.5-, 1.2-, 1.6-, and 5.8-fold increases in vBMD, BV/TV, Tb.N, Tb.Th, and stiffness, respectively, with a 23% decrease in SMI.

Sost *KO* mice are well known for their high bone mass phenotype. Consistent with this, we observed significant increases in trabecular vBMD (3-fold), BV/TV (3.1-fold), Tb.Th (2.1-fold), and stiffness (13.7-fold) in these age- and sex-matched *KO* mice compared with

vehicle-treated non-irradiated *WT* bones (Fig. 2). *KO* animals also exhibited a significant reduction in SMI to nearly 0. Interestingly, except for the slightly elevated Tb.Th and SMI, there was no significant difference in all other bone structural parameters observed between the radiated and non- radiated femurs in the same *KO* mice. Taken together, our data provide the first in vivo evidence that suppressing sclerostin action is effective in preventing radiation-induced bone damage.

Suppressing sclerostin stimulates bone formation and inhibits bone resorption after radiation

Immediately after the second radiation (day 3), we observed remarkable vessel swelling and erythrocyte leakage accompanied by a highly significant decrease in bone marrow cellularity (Supplemental Fig. S2A, B). Marrow adiposity appeared at day 3 and substantially increased at day 8 (Supplemental Fig. S2B, C). Hematopoietic components started to recover by day 8. At 2 weeks post-radiation, bone marrow cellularity and hematopoietic cell populations in radiated metaphyseal area were already close to those in non-radiated area (Supplemental Table S2). Interestingly, we observed a significant increase in CD41⁺ platelets at this stage, coinciding with their important role in repairing damaged vessels.⁽³⁰⁾ By 4 weeks post-radiation, most marrow abnormalities had disappeared except marrow adiposity (Fig. 3A). This is in contrast to our previous report that rat bone marrow had not recovered at 4 weeks after radiation,⁽¹⁵⁾ suggesting a species-specific response to radiation.

To understand how Scl-Ab treatment rescues bone damage after radiation, we performed bone histomorphometry on bilateral femurs from vehicle- and Scl-Ab-treated *WT* and *Sost KO* mice after radiation. Radiation caused detrimental effects on osteoblasts with a 52% reduction in osteoblast number (Ob.N/BS) compared with contralateral non-radiated femurs (Fig. 3B). Dynamic analysis revealed no calcein double-labeled surfaces in vehicle-treated radiated femurs, leading to no measurement value for bone formation rate (BFR) (Fig. 3C, D). Moreover, mineralizing surface (MS) was greatly reduced by 6.9-fold in the irradiated bone (Fig. 3C, D). Suppressing sclerostin by either exogenous Scl-Ab injections or endogenous gene *KO* partially attenuated the increased marrow adiposity after radiation (Fig. 3A). Moreover, in both animal models, osteoblast number and activity were remarkably increased after radiation compared with vehicle-treated radiated *WT* femurs (Fig. 3B–D). Note that these increases have not yet reached the same level as non-radiated left femurs, indicating that the damaging effects of radiation on osteoblasts cannot be fully reversed by Scl-Ab treatment. These histological changes were further confirmed by elevated serum levels of bone formation marker (osteocalcin) in both Scl-Ab-treated *WT* and *Sost KO* mice (Fig. 3E).

Our radiation protocol did not inhibit bone resorption. We did not observe significant changes of osteoclast surface (Oc.S/BS) between irradiated and non-irradiated vehicle-treated *WT* bones (Fig. 3B). Furthermore, suppressing sclerostin by either Scl-Ab injection or *Sost KO* significantly decreased osteoclast surface with a greater effect in the latter case. Similarly, bone resorption marker, serum CTX-I level, was significantly decreased in both (Fig. 3E).

Analysis of mesenchymal lineage cells in *Col2/Tomato* mice

To delineate the cellular mechanisms by which Scl-Ab injections modulate bone-forming cells after radiation, we utilized a lineage tracing model, *Col2/Tomato* mice, in which cells expressing Cre recombinase driven by the *Col2* promoter contribute to a majority of osteoblasts, Cxcl12-abundant stromal cells, and bone marrow stromal/mesenchymal progenitor cells in postnatal life.⁽³¹⁾ Most importantly for our studies, we observed similar radiation damage to the trabecular bone and similar rescue effects of Scl-Ab treatment in these mice as with *WT* mice (data not shown).

To validate this model, we first confirmed that almost all osteoblasts and osteocytes in the trabecular bone (Fig. 4A, B), endosteal osteoblasts on the cortical bone, and osteocytes in the cortical bone (Fig. 4A, C) are Tomato⁺ in the long bones of *Col2/Tomato* mice. Within bone marrow, all perilipin⁺ adipocytes were Tomato⁺ (Fig. 4D). Interestingly, some perivascular cells were also Tomato⁺ (Fig. 4E), suggesting that they are likely to be MSCs. Scattered mononuclear, non-adipocytic Tomato⁺ cells were visible inside the entire bone marrow, with more occurrences in the metaphyseal region than in the diaphyseal region. We previously developed an enzymatic digestion method to release bone marrow cells trapped within the trabecular bone (endosteal bone marrow)⁽³²⁾ and proved that they contain a higher frequency of mesenchymal progenitors than central bone marrow, which is flushed from diaphysis. Using this approach, we found that the frequency of Tomato⁺ cell in endosteal bone marrow is about 1.03% and these cells are mostly CD45⁻CD31⁻ (>95%, Fig. 4F). When endosteal bone marrow cells were cultured for CFU-F assay, more than 90% of the resulting colonies were Tomato⁺ (Fig. 4G) and sorted Tomato⁻ endosteal bone marrow cells did not form colonies (data not shown). After osteogenic or adipogenic differentiation, these cultured mesenchymal progenitors formed Tomato⁺ bone nodules (Fig. 4H) and adipocytes (Fig. 4I). These data clearly demonstrate that Tomato marks almost all mesenchymal lineage cells in endosteal bone marrow but not non-mesenchymal cells, such as hematopoietic cells and endothelial cells. Therefore, *Col2/Tomato* mice represent a suitable model for examining bone marrow mesenchymal progenitors in vivo.

Scl-Ab protects osteoblasts from radiation-induced apoptosis by accelerating DNA repair

At 2 weeks after radiation, TUNEL staining of irradiated bone revealed a 3.9-fold increase in the number of apoptotic osteoblasts on bone surface (Fig. 5A, B). Weekly Scl-Ab treatment significantly reduced the number of apoptotic osteoblasts, thereby protecting the functional osteoblasts. In non-irradiated bones, Scl-Ab also displayed a tendency of reducing the number of apoptotic osteoblasts.

Upon radiation, the cell fate is determined by the magnitude of DNA damage to the cells and the DNA repair ability of the cells. Radiation induces highly lethal DNA lesions, among which DSB is considered to be the major cause of cell death, because unrepaired or misrepaired DSBs can lead to genomic instability and cell death. In mammalian cells, the major repair pathway for DSBs is the NHEJ pathway.⁽³³⁾ Classic NHEJ first recognizes and binds to broken ends by the Ku70/80 heterodimer, which subsequently activates the DNA-dependent kinase complex (DNA-PKcs) and recruits other factors to prepare the DNA ends for ligation by the DNA ligation complex. Our previous in vitro data demonstrated that

activation of the canonical Wnt pathway accelerates DNA repair and protects osteoblastic cells from radiation-induced cell death via increased Ku70 amount.⁽¹⁷⁾ Fluorescent immunostaining revealed a similar mechanism in vivo. At 2 weeks after radiation, bone surface Tomato⁺ osteoblasts exhibited 2.8-fold more γ -H2AX foci, a marker for DSB, than those in non-irradiated bones (Fig. 5C, D). Scl-Ab treatment greatly attenuated this increase to a comparable level of those in non-irradiated bones. Furthermore, although Ku70 immunostaining in Tomato⁺ osteoblasts was very low under control, radiation alone, or Scl-Ab alone conditions, it was substantially elevated (4.9-fold compared with control) after radiation and Scl-Ab injections (Fig. 5E, F). Together, these in vivo results prove that Scl-Ab protects osteoblasts from cell death after radiation by accelerating DNA repair in osteoblasts.

Scl-Ab improves osteocyte survival and their canalicular network after radiation

Focal radiation resulted in a significant increase in the number of empty lacunae at 4 weeks (Fig. 6A, B). Compared with osteoblasts, osteocytes seem to be less sensitive to radiation because the percentage of TUNEL⁺ osteocytes was lower than TUNEL⁺ osteoblasts at 2 weeks (Fig. 6C). Similar to osteoblasts, this radiation-induced osteocyte apoptosis was almost completely abolished by concomitant Scl-Ab treatment (Fig. 6A–C). Interestingly, we also observed an increase in γ -H2AX foci in osteocytes and this increase was attenuated by Scl-Ab, indicating a similar DNA repair mechanism by Scl-Ab in stimulating osteocyte survival (Fig. 6D).

Osteocytes have a vast network of long, dendritic-like cell processes for communication with each other and with bone surface cells via connexin43-containing gap junctions.⁽³⁴⁾ This osteocytic canalicular network is also important for maintaining osteocyte function and cortical bone quality. After staining with fluorophore-labeled phalloidin to detect F-actin, normal canaliculae were observed in non-irradiated trabecular bone with robust inter-osteocytic and osteocyte-bone surface connectivity. Exposure to focal radiation caused a 42% reduction in the total canalicular length per osteocyte, resulting in a disruption of the osteocytic network (Fig. 6E, F). Scl-Ab had no obvious effects in non-irradiated samples but strikingly blocked the decrease of canalicular connection after radiation, further supporting the idea that Scl-Ab protects osteocytes from radiation damage.

Scl-Ab prevents radiation-induced cell fate shift of bone marrow mesenchymal progenitors but not their loss of proliferation potential

Bone marrow mesenchymal progenitors are the multipotent progenitors for osteoblasts and adipocytes in bone. The traditional approach to measure their frequency is through CFU-F assay. With this approach, we demonstrated a more than 93% reduction in CFU-F colony number in endosteal bone marrow in vehicle-treated *WT* mice at 2 weeks after radiation (Fig. 7A). Suppressing sclerostin either by Scl-Ab injection or by *Sost* *KO* modestly increased the CFU-F colony frequency in both non-irradiated and irradiated bones. Note that even after suppressing sclerostin, CFU-F frequencies of irradiated endosteal bone marrow were still much lower than those of non-irradiated cells (Fig. 7A), suggesting that activating the Wnt canonical pathway cannot completely block radiation damage on mesenchymal progenitors.

Although the CFU-F assay has been commonly used in bone research, it does not accurately reflect the *in vivo* situation because by nature it is an *in vitro* assay. To measure the number of endosteal mesenchymal progenitors, we quantified Tomato⁺ pericytes and non-adipocytic stromal cells within the metaphyseal long bone region of *Col2/Tomato* mice. Interestingly, we found that focal radiation only moderately decreases Tomato⁺ pericytes and stromal cells by 40% and 31%, respectively (Fig. 7B, C). Note that those numbers are drastically different from the percentage decrease after radiation observed in the CFU-F assay. Meanwhile, the number of Tomato⁺perilipin⁺ bone marrow adipocytes measured within the same area was substantially elevated after radiation (Fig. 7B, C). Again, after radiation, all perilipin⁺ bone marrow cells were Tomato⁺, suggesting that newly formed adipocytes originate from mesenchymal progenitors. When we added these three populations of Tomato⁺ bone marrow cells together, it was evident that the total number of Tomato⁺ bone marrow cells did not change substantially after radiation (Fig. 7D). Moreover, we did not detect any TUNEL⁺Tomato⁺ pericytes and stromal cells or γ -H2AX foci within these cell populations. Therefore, these results strongly indicate that the major radiation damage to mesenchymal progenitors is not cell death, as originally implied by the CFU-F assay, but is cell fate shift, by directing most progenitors toward adipocyte differentiation. Indeed, we observed that there was a surge in the number of perivascular adipocytes immediately after radiation (Supplemental Fig. 2D, E), implying that perivascular MSCs differentiate quickly into adipocytes. Interestingly, Scl-Ab treatment was beneficial by almost completely blocking this cell fate shift (Fig. 7B–D). qRT-PCR analysis further confirmed that radiation increases the expression of several adipogenic markers, such as PPAR γ , LPL, and aP2 in bone and that Scl-Ab treatment attenuates such increases (Supplemental Fig. S3). Consistent with the notion that radiation does not cause apoptosis in bone marrow mesenchymal progenitors, we also did not detect any Ku70 staining in those cells after Scl-Ab treatment and radiation.

To further understand the large difference between the percentage decreases in the number of mesenchymal progenitors after radiation, as assayed by CFU-F and by *in vivo* quantification, we irradiated endosteal mesenchymal progenitors in culture. Unlike osteoblastic cells that normally go through apoptosis after radiation (12.6% in irradiated cells versus 1.0% in control), mesenchymal progenitors displayed undetectable levels of cell death at 2 days after radiation (Fig. 7E). However, the colony-forming ability of those irradiated progenitors was severely damaged, leading to a 91% decrease in CFU-F numbers compared with non-irradiated progenitors after they were immediately reseeded (Fig. 7F, G), implying that radiation causes a loss of proliferation potential in those progenitors. Wnt3a, a canonical Wnt ligand, had no rescue effects on this radiation damage. These data explain why *in vivo* quantification of Tomato⁺ cells detects a much milder decrease in the number of mesenchymal progenitors after radiation compared with CFU-F assay, as those progenitors, which were still alive *in vivo*, were irreversibly growth arrested when cultured in the growth medium.

Discussion

Exposure to ionizing radiation, despite adherence to standard protocols and dosages, collapses bone marrow hematopoietic components, devastates marrow vasculature, and compromises bone architecture and strength. Patients undergoing radiotherapy develop

elevated fracture risks at the irradiated sites. Therefore, it is important to understand the mechanisms of radiotherapy-induced osteoporosis that can guide the development of appropriate treatments. In the present study, we first confirm our previous conclusions^(15,16) that bone damage resulting from clinically relevant focal radiation is mainly due to its inhibitory effects on bone formation, rather than an overactivation of bone resorption. This cellular mechanism reinforces the concept that any effective therapy must be directed at preserving bone-forming activity than suppressing bone resorptive activity. Next, we demonstrate that Scl-Ab treatment, a yet to be approved anabolic drug for postmenopausal patients, is effective in alleviating radiation-induced bone loss by protecting mesenchymal progenitors, osteoblasts, and osteocytes via distinct mechanisms. In line with a recent report that mouse serum sclerostin level is significantly elevated at 1 month after total body irradiation,⁽³⁵⁾ we found that focal radiation increases sclerostin protein in osteocytes. Our current Scl-Ab treatment is a preventive protocol that suppresses sclerostin activity at the initiation of radiotherapy. Importantly, the bone structure of *Sost KO* mice, which lacks sclerostin long before the radiation, is insensitive to radiation. These data substantiate our conclusion that Scl-Ab is a potential therapy for radiation-associated bone loss.

Our early studies indicated that rhPTH1-34 could be used as a treatment for radiation-induced bone damage because of its strong radioprotective effects on osteoblasts.^(15–17) However, owing to preclinical data showing an increased incidence of osteosarcoma in rats treated with high-dose rhPTH1-34,⁽³⁶⁾ the FDA approval of this agent carries a black box warning against use in patients with cancer or bone radiation. Hence, PTH is unlikely to be used in cancer patients despite a decade of safe clinical use of PTH1-34 and full-length 1-84 in patients with osteoporosis.^(37,38) Because Wnt/ β -catenin signaling is a regulatory pathway involved in cell proliferation and differentiation, its potential overactivation leading to tumor recurrence or new tumorigenesis is also of concern. It is important to note that sclerostin expression is strictly limited to osteocytes^(21,22) and hypertrophic chondrocytes.⁽³⁹⁾ Patients with sclerosteosis (loss-of-function mutations in *Sost* gene), van Buchem disease (a deletion of genomic DNA downstream of *Sost* gene), or *LRP5* high bone mass disorder (gain-of-function mutations that disrupt LRP5 binding with sclerostin and DKK1) do not have increased cancer incidence despite a small population size.⁽⁴⁰⁾ *Sost KO* mice have a normal life span with no increased tumor formation. Indeed, past years of animal studies and human clinical trials of Scl-Ab have revealed no carcinogenic effect. Moreover, there is a so-called “self-regulation” mechanism existing with longer-term Scl-Ab treatment in which bone formation and osteoblast activity return to control levels after 12 weeks of treatment.⁽⁴¹⁾ Interestingly, longer-term treatment of Scl-Ab increases P53, a tumor suppression marker, in osteoblast lineage cells.⁽⁴²⁾ An additional benefit of Scl-Ab is that, unlike PTH, which activates bone remodeling, Scl-Ab mainly stimulates modeling-based formation while modestly inhibiting resorption.⁽⁴³⁾ This might block cancer metastasis to bone, which requires bone resorption to release tumor-promoting factors. Phase 2 clinical trials of Scl-Ab (romosozumab and blosozumab) showed that these antibodies are well tolerated in patients with the only noticeable side effect of mild injection site reactions.^(24,44) Therefore, activating the Wnt canonical pathway via Scl-Ab is more clinically acceptable than PTH to provide much-needed, yet currently unavailable, therapy for damaged bone in cancer patients undergoing radiotherapy. That said, it will be necessary to perform extensive

preclinical studies and clinical trials to determine the benefits and risks of a short-term Scl-Ab treatment in combination with radiotherapy in cancer patients.

After demonstrating its *in vivo* efficacy, we set out to investigate the underlying mechanism by which Scl-Ab preserves bone formation through mesenchymal lineage cells. Using a lineage tracing approach recently reported by Ono and colleagues,⁽³¹⁾ we demonstrated that the Tomato signal in *Col2/ Tomato* mice marks all mesenchymal cells, including perivascular MSCs, stromal cells, adipocytes, osteoblasts, and osteocytes, in endosteal bone marrow but not hematopoietic cells or endothelial cells. This model allows us, for the first time, to visualize and quantify bone-forming cells, especially mesenchymal progenitors including MSCs and non-adipocytic stromal cells, *in vivo*. We observed that focal radiation induces more cell death in osteoblasts than in osteocytes through damaging DNA as revealed by γ -H2AX staining. This is conceivable because osteocytes have much longer life (years) than osteoblasts (months in human). Consistent with our previous cell culture study using Wnt3a as an activator,⁽¹⁷⁾ activating Wnt/ β -catenin by Scl-Ab treatment increased Ku70 level, enhanced DNA repair, and thus promoted survival in these terminally differentiated cells. While completely encased within a mineralized extracellular matrix, osteocytes develop an extensive canalicular network for intracellular and extracellular communication, including their communication to bone surface osteoblasts. This functional network not only allows for passage of nutrients and biochemical signals but is also ideal for mechanotransduction.⁽³⁴⁾ Recent studies also indicate that this canalicular structure is the major determinant of bone matrix mineralization.^(45,46) Interestingly, we found that Scl-Ab treatment rescues the radiation damage to this network, thus allowing osteocytes to maintain their normal highly dendritic morphology and physiological functions. Whether this rescue effect of Scl-Ab on osteocytic connectivity is correlated with its survival effect is not known at this point but is worth further investigation.

In contrast to its action on osteoblasts and osteocytes, radiation does not seem to destroy their progenitors directly *in vivo*. We never detected TUNEL and/or γ -H2AX signals in Tomato-labeled bone marrow mesenchymal progenitors. One possible explanation could be that apoptotic progenitors quickly disappear from bone marrow; for example, by macrophage consumption. However, when bone marrow Tomato⁺ cells were counted, we found that the total number of bone marrow MSCs, stromal cells, and adipocytes remained the same, regardless of radiation and Scl-Ab treatment, but that the percentage of each population was altered. Radiation preferentially induces adipogenesis in both MSCs and stromal cells, leading to shrinkage of the progenitor population. Interestingly, we (data not shown) and others^(47,48) have found that *in vitro* radiation does not directly accelerate the adipogenic differentiation of cultured MSCs, indicating that this is not a cell-intrinsic mechanism. How radiation causes marrow adiposity *in vivo* is still largely unknown. Because focal radiation causes immediate and sustained increase of free radicals in bone marrow⁽⁴⁹⁾ and oxidative stress, in which ROS generation outpaces the antioxidant system, is a major stimulant of MSC adipogenesis,⁽⁵⁰⁾ one intriguing possibility is that radiation shifts the fate of mesenchymal progenitors toward adipocytes by elevating their environmental ROS level. Activation of Wnt/ β -catenin is well known to inhibit key transcriptional factors C/EBP α and PPAR γ for adipogenesis in mesenchymal progenitors.⁽⁵¹⁾ Thus, Scl-Ab treatment is able to partially block the radiation damage to

mesenchymal progenitors by preventing their adipogenic differentiation. Marrow adipocytes are not only a space filler but also have negative impacts on bone marrow hematopoietic microenvironment.⁽⁵²⁾ Like PTH,⁽¹⁵⁾ Scl-Ab does not change radiation-induced short-term marrow collapse in mice (data not shown). Because of its effects on marrow adiposity, it would be interesting to further study whether it has beneficial effects on the recovery of hematopoietic cells after radiation.

Surprisingly, although our CFU-F data and finding by other investigators⁽⁴⁹⁾ suggest that high-dose focal radiation eliminates the majority of bone marrow mesenchymal progenitors, our *in vivo* counting in *Col2/ Tomato* mice revealed only a 34.5% decrease in the number of MSCs and stromal cells, which was attributable to increased adipogenesis. Further *in vitro* experiments revealed that, unlike osteoblasts, MSCs do not undergo apoptosis after radiation but instead lose their proliferation potential. This explains why very few colonies are formed when they were expanded in the CFU-F assay. It is possible that if given longer time to allow the disappearance of radiation-arrested mesenchymal progenitors, the results of CFU-F assay and *in vivo* counting of Tomato⁺ progenitors would become similar. We found that activation of Wnt/ β -catenin signaling cannot rescue this type of radiation damage to mesenchymal progenitors. This raises the issue of whether Scl-Ab treatment could preserve long-term bone-forming activity at irradiated sites. Because MSCs are capable of mobilizing to the injured sites and participating in the tissue regeneration process,^(53,54) we believe that MSC residing in non-irradiated bone may aid in long-term bone recovery after radiation. Furthermore, co-administration of healthy MSCs could be an option to enhance the efficacy of Scl-Ab in treating radiotherapy-induced osteoporosis.

In summary, our studies provide the first proof-of-principle evidence for a novel use of Scl-Ab as a treatment for radiation-induced osteoporosis and the delineation of molecular and cellular mechanisms to support such treatment. A recent report points out that a bispecific antibody targeting both sclerostin and DKK1 shows superior bone repair activity compared with monotherapy.⁽²⁹⁾ Thus, it would be interesting to test the therapeutic effects of this dual-action antibody in our focal radiation model. Our long-term goal is to benefit millions of cancer patients by developing a therapy that will eventually allow the use of maximal radiotherapy doses on tumors while at the same time preventing the potentially severe bone-associated side effects. Further experiments to optimize the treatment regimen and dosage, to better mimic the clinical scenario by co-administering other cancer therapies, such as chemotherapy, and to address the theoretical cancer concerns of activating Wnt/ β -catenin signaling are required before translating this treatment into a clinical application.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Focal radiation increases the amount of sclerostin in bone. (A) IHC of sclerostin (brown color) in trabecular bone from bilateral femurs of *WT* mice treated with either vehicle or Scl-Ab for 2 weeks. Bone marrow cells are shown as green and bone matrix are white. (B) Quantification of the relative intensity of sclerostin staining in trabecular osteocytes. $n = 3$ femurs/group. NR = non-radiated femur; R = radiated femur. $a = p < 0.05$ R versus NR.

Fig. 2. Inhibition of sclerostin reverses trabecular bone loss and strength deterioration caused by SARRP radiation. (A) 3D reconstructed μ CT images of trabecular bone harvested from *WT* mice treated with either vehicle or Scl-Ab injections and from *Sost* *KO* mice at 4 weeks after SARRP radiation. (B) μ CT measurement of trabecular bone structural parameters. $n = 7$ femurs/group. (C) Trabecular bone stiffness was measured using μ FE model. a = $p < 0.05$; b = $p < 0.01$; c = $p < 0.001$ R versus NR; * = $p < 0.05$; # = $p < 0.01$; \$ = $p < 0.001$ Scl-Ab versus veh or *KO* versus *WT*.

Fig. 3.

Suppression of sclerostin greatly stimulates bone formation and inhibits bone resorption after radiation. (A) Goldner's trichrome staining of distal femoral trabecular sections from *WT* mice treated with either vehicle (veh) or Scl-Ab and from *Sost KO* mice at 4 weeks after SARRP radiation. (B) Static histomorphometric analysis quantifying osteoblast number (Ob.N/BS) and osteoclast surface (Oc.S/BS). $n = 5$ femurs/group. (C) Representative calcein and xylenol orange double labeling in the trabecular bone. (D) Dynamic histomorphometric analysis quantifying BFR and MS. $n = 5$ femurs/group. (E) ELISA analyses of serum bone formation (osteocalcin) and resorption (CTX-I) markers in whole animals. $n = 7$ mice/group. Ab = Scl-Ab. a = $p < 0.05$; b = $p < 0.01$ R versus NR; * = $p < 0.05$; \$ = $p < 0.01$; # = $p < 0.001$ Scl-Ab versus veh or *KO* versus *WT*.

Fig. 4.

Bone marrow non-adipocytic Tomato⁺ cells are mesenchymal progenitors in *Col2/Tomato* mice. (A) Fluorescence image of longitudinal femoral sections from 1-month-old *Col2/Tomato* mice with DAPI staining (blue). Red = Tomato. (B, C) Magnified views showing Tomato expression in trabecular bone (B) and cortical bone (C). (D) Representative images of bone marrow adipocytes (arrows) marked by both perilipin (white) and Tomato (red). (E) Some Tomato⁺ cells (pointed by arrows) are perivascular cells. Green = Endomucin staining for endothelial cells. (F) Flow cytometry demonstrates that Tomato⁺ endosteal bone marrow cells are CD31⁻CD45⁻. (G) The CFU-F colonies derived from endosteal bone marrow contain mostly Tomato⁺ cells. (H) Almost all cells surrounding bone nodules formed in osteogenically differentiated culture of endosteal mesenchymal progenitors are Tomato⁺ cells. (I) Similarly, almost all adipocytes (identified by lipid droplets) from adipogenically differentiated culture are Tomato⁺.

Fig. 5.

Scl-Ab treatment accelerates DNA repair and attenuates apoptosis in osteoblasts caused by focal radiation. (A) TUNEL staining revealed that 2 weeks of Scl-Ab treatment reverses the radiation-induced osteoblast apoptosis (green, pointed by yellow arrowheads) in the trabecular bone. Dashed lines depict trabecular bone surfaces. (B) Quantification of TUNEL⁺ osteoblasts within the metaphysis. (C) Immunofluorescent staining of γ -H2AX foci (white, pointed by yellow arrows) in trabecular osteoblasts after either vehicle or Scl-Ab treatment 2 weeks after radiation. (D) Quantification of the number of γ -H2AX foci per osteoblast. (E) Immunofluorescent staining of Ku70 (green, pointed by white arrows) in trabecular osteoblasts after either vehicle or Scl-Ab treatment 2 weeks after radiation. (F) Quantification of the percentage of Ku70⁺ osteoblasts. $n = 3$ femurs/group. $a = p < 0.05$; $c = p < 0.001$ R versus NR; $\$ = p < 0.01$; $\# = p < 0.001$ Scl-Ab versus veh.

Fig. 6.

Scl-Ab treatment blocks radiation damage to osteocytes. (A) Goldner's trichrome staining revealed that Scl-Ab injections mitigate radiation-induced empty lacunae (indicated by red arrows) in the trabecular bone. (B) Quantification of the number of empty lacunae at 4 weeks after radiation. BA = bone area. $n = 5$ femurs/group. (C) Comparison of the percentages of TUNEL⁺ osteoblasts and osteocytes at 2 weeks post-radiation. $n = 3$ femurs/group. (D) Scl-Ab attenuates the increase of osteocytes with γ -H2AX foci caused by focal radiation. $n = 3$ femurs/group. (E) Fluorescent phalloidin staining showed that Scl-Ab reverses radiation damage on osteocytic canalicular network (pointed by white arrows) at 2 weeks. (F) Quantification of total canalicular length (CL.L.) per osteocyte. $n = 3$ femurs/group. $a = p < 0.05$; $c = p < 0.001$ R versus NR. $^* = p < 0.05$ Scl-Ab versus veh.

Fig. 7.

Scl-Ab prevents radiation-induced cell fate shift of bone marrow mesenchymal progenitors but not their growth arrest. (A) CFU-F assays were performed with endosteal bone marrow cells from irradiated bone and non-irradiated contralateral ones at 2 weeks after radiation in *WT* mice with or without Scl-Ab treatment and in *Sost KO* mice. $n = 4$ femurs/group. (B) Representative fluorescence immunohistologic images show the changes of bone marrow Tomato⁺ cells (pericytes, white arrowheads; adipocytes, yellow asterisks) in *Col2/Tomato* mice after radiation and Scl-Ab treatment. (C) Quantification of bone marrow Tomato⁺ cells, including pericytes, non-adipocytic stromal cells, and adipocytes. BMA = bone marrow area. $n = 3$ femurs/group. (D) The quantification data in C are summarized in one graph to show that radiation and Scl-Ab do not change the overall number of bone marrow Tomato⁺ cells but alter their differentiation fate. (E) Radiation induces apoptosis in osteoblastic UMR106 cells but not in endosteal mesenchymal progenitor (MP) culture as revealed by EB/AO staining (arrows). (F) Endosteal mesenchymal progenitors were irradiated and reseeded for colony formation assay in the absence or the presence of Wnt3a. Colonies were stained after 2 days. (G) The numbers of colonies formed were quantified. $n = 3$ /group. $a = p < 0.05$; $c = p < 0.001$ R versus NR. $* = p < 0.05$; $\$ = p < 0.01$; $\# = p < 0.001$ Scl-Ab versus veh.