Activating Canonical Wnt/β-catenin Pathway Enhances DNA Repair and Promotes Cell Survival in Osteoblasts: a Novel Anabolic Treatment for Radiotherapy Associated Bone Damage

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Introduction: A preventive or curative treatment for bone damage caused by radiation therapy targeting neighboring tumor is still elusive. Understanding the mechanisms behind these adverse effects of radiation and exploring new treatments for such bone disorders is imperative to improve the quality of life for these cancer patients. We recently established a clinically relevant radiation model using skeletally mature rats and a newly available small animal radiation research platform (SARRP) that replicates focal clinical radiotherapy in rodents. With this model, we reported that focal radiation on rat bone causes a loss of small trabecular elements with decreases in bone mass and strength and that diminished bone formation, but not enhanced bone resorption is a major contributor to such bone loss [1]. Interestingly, daily injections of parathyroid hormone (PTH1-34, teriparatide), a FDA-approved anabolic treatment for severe osteoporosis, blocks post-radiation bone damage via protecting osteoblasts and osteocytes from apoptosis [1]. Further cell culture studies delineated that the survival effect of PTH is mediated through a PKA/β-catenin pathway. In this study, we investigated the molecular mechanism by which activating canonical Wnt/β-catenin pathway protects osteoblasts from radiation damage and explored whether Sclerostin antibody (Scl-Ab), a treatment that specifically stimulates β-catenin activity in bone, is efficient in preventing radiation induced-bone damage.

Methods: Radiation on osteoblast lineage cells and Wnt3a treatment- Xrad 320i was used to deliver a dose of 8 Gy for osteoblastic (UMR106-01) and osteocytic (Ocy454) cells and 24Gy for primary calvarial osteoprogenitors. Wnt3a conditioned medium was collected from Wnt3a overexpressing L cells and applied to osteoblast lineage cells at 30 min after radiation. Cell death detection- Ethidium Bromide (EB)/Acridine Orange (AO) staining was used for detection of apoptotic cells. Immunofluorescence- After radiation, cells were fixed with 4% paraformaldehyde and incubated with antibodies against γ-H2AX, caspase 3 and Ku70 followed by Alexa-conjugated fluorescent secondary antibodies and DAPI staining. Single cell gel electrophoresis- To measure the extent of DNA damage at a single cell level, comet assay was performed using the alkaline conditions of the Trevigen Comet Assay® kit. SARRP radiation and Scl-Ab treatment in mice- Two-month-old male C57BL6 (n=7/group) received a total of 16 Gy radiation, fractionated as two 8 Gy doses delivered on days 1 and 3 to the distal metaphyseal region of the right femurs using SARRP (Xstrahl, Suwanee, GA). This was designed to mimic the typical femur dose constraints for whole pelvis intensity modulated radiotherapy for patients with prostate, rectal, or endometrial cancers. Following radiation, mice were intraperitoneally injected with either vehicle or Scl-
Ab (100mg/kg/week) for 4 weeks. µCT- Bilateral femurs were harvested on d28 for µCT measurement of trabecular structural parameters and stiffness based on finite-element modeling.

**Results:** To study the survival effect of Wnt signaling on osteoblast lineage cells, UMR106-01, Ocy454, and primary osteoprogenitor cells were radiated and cultured in Wnt3a-containing medium. Apoptosis assay using either EB/AO (Fig. 1A-C) or cleaved caspase-3 staining (Fig. 1D, E) revealed that Wnt3a strongly suppressed radiation-induced cell death in those cells. This survival effect relies on the canonical pathway because both IWR-endo (Fig. 1A), an inhibitor for the canonical β-catenin pathway, and β-catenin siRNA (Fig. 1B), were able to completely abolish this effect. Radiation induces highly lethal DNA damage, among which double strand break (DSB) is the major cause of cell death. Two sensitive methods to detect DSBs are immunofluorescence staining of γ-H2AX and comet assay at a single cell level. We found that Wnt3a attenuated the number of nuclear γ-H2AX foci (Fig. 2A, B) and reduced the amount of tail (damaged) DNA (Fig. 2C, D) in UMR cells after radiation. In mammalian cells, most radiation-induced DSBs are repaired through non-homologous end-joining (NHEJ) pathway. After radiation, Wnt3a increased the amount of Ku70 (Fig. 2E), a critical first component of NHEJ pathway, via β-catenin pathway in osteoblasts in a time-dependent manner. Pre-treatment with 500ng/ml Dkk1 could abolish the Wnt3a effect on Ku70 expression (Fig. 2F). Interestingly, knocking down the expression of Ku70 strongly abolished the DNA repair and cell survival actions of Wnt3a on osteoblasts (Fig. 2G-I). Taken together, our data clearly demonstrate that activating the Wnt/β-catenin pathway enhances DNA repair and therefore protect osteoblast lineage cells from radiation damage. To translate these in vitro findings into in vivo studies, we focally irradiated adult mice at distal femoral metaphysis followed by weekly injection of Scl-Ab for 4 weeks. As shown in Fig. 3, 16 Gy radiation generated from SARRP induced a significant trabecular bone loss and structural deterioration in irradiated femurs compared to contralateral ones in vehicle-treated mice (BMD: -20%; BV/TV: -21%; Tb.N: -10%; SMI: +30%; Stiffness: -75%). Remarkably, Scl-Ab injections increased trabecular BMD, BV/TV, Tb.N, decreased SMI and increased stiffness to a similar level regardless of radiation, implying that Scl-Ab treatment is able to reverse radiation-induced bone damage in a clinical setting.

**Discussion:** This study provides proof-of-principle evidence that anabolic therapy is efficient for radiation-induced osteoporosis. Radiation damage on bone is mainly caused by its genotoxic damage and subsequent cell death in osteoblast lineage cells. Our in vitro studies delineate that one underlying mechanism for the survival effect of activating Wnt/β-catenin pathway is through up-regulation of Ku70 and accelerating the repair of DSBs. Subsequent animal study demonstrate that injections of Scl-Ab are capable of blocking bone loss and microarchitecture deterioration after radiation.

**Significance:** Fractures in tumor neighboring bones is a significant side effect of radiotherapy that poses major health threats for those cancer patients, most of them are elderly and with a greater risk of age related osteoporosis. Scl-Ab is currently under clinical trial for postmenopausal osteoporosis and could be an alternative and better treatment for radiotherapy-induced bone loss compared to PTH1-34 because of its black box warning.
Figure 1. Wnt protects osteoblasts from radiation-induced apoptosis through canonical β-catenin pathway.
(A) Quantification of EB/AO staining of UMR cells at 2 days after radiation. (B) Cells transfected with or without siRNA against β-catenin were radiated and treated with or without Wnt3a-CM. Apoptotic cells were quantified. (C) Cells treated as in (B). (D) Quantification of apoptotic rat calvaria osteoprogenitors (left) and osteocytes (right) after radiation and Wnt3a treatment. (E) Immunofluorescence of cleaved-caspase3 after radiation and Wnt3a treatments. (F) Quantification of cells with nuclear cleaved caspase3+ NR: non-radiated; R: radiated. ∆: p<0.001 vs NR; ∆: p<0.001 vs R; ∆: p<0.01; ∆: p<0.001 vs R+Wnt.
Figure 2. The canonical Wnt-signaling promotes DNA repair in radiated osteoblastic cells.
(A) Immunofluorescence images of γ-H2AX foci in UMR cells after radiation. (B) Quantification of γ-
H2AX foci number after radiation with or without Wnt3a and β-catenin siRNA(C, D) Comet assay
shows amount of damaged DNA in UMR cells with or without siRNA against β-catenin. &: p<0.001 vs
NR; δ: p<0.001 vs R; Σ: p<0.001 vs R-Wnt-mock-siRNA. (E, F) Immunoblots show that Wnt3a
increases Ku70 amount in UMR cells via β-catenin pathway. (G-I) siRNA against ku70 was transfected
in UMR cells followed by radiation and treatment with Wnt. γ-H2AX foci number (G), damaged tail
DNA (%) (H) and cell apoptosis (I) were quantified. &: p<0.001 vs NR; δ: p<0.001 vs R; Σ: p<0.01; ♦:
p<0.001 vs Wnt mock-si-RNA.
Figure 3. Scl-Ab reverses SARRP radiation-induced trabecular bone loss and strength deterioration.
A. Representative 3D images of distal femoral trabecular bones at day 28 after radiation with or without Scl-Ab treatment.
B. μCT measurement of bone structural parameters and bone strength. a: p<0.05; b: p<0.01; c: p<0.001 R vs NR; *: p<0.05; #: p<0.01; $: p<0.001 Scl-ab vs veh.

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