UNIVERSITY OF CALIFORNIA SAN DIEGO

Activation of NO/cGMP/PKG Signaling Reduces Bone Loss in Mice with Senile Osteoporosis

A thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

Biology

By Jafar Moininazeri

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Professor Keefe D. Reuther

2022

The thesis of Jafar Moininazeri is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

University of California San Diego

2022

DEDICATION

To my mother Rosie, my grandmother Tutu, and my aunt Isabel, thank you for always supporting my dreams, encouraging my ambitions, and inspiring me to push myself. To my better-half Mercedes, I couldn't have done it without all your love and support. Thank you for always believing in me and keeping me focused.

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ABSTRACT OF THE THESIS

Activation of NO/cGMP/PKG Signaling Reduces Bone Loss in Mice with Senile Osteoporosis

By

Jafar M. Moininazeri

Master of Science in Biology

University of California San Diego, 2022

Professor Gerard R. Boss, Chair

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Excessive bone loss due to aging is a complication of senile osteoporosis, a severe skeletal disorder causing bones to become weaker and more susceptible to fractures over time. Current therapeutics to treat osteoporosis such as bisphosphonates, hormone replacements and nitric oxide donors proved ineffective as these drugs provoke serious health complications associated with increased oxidative stress and cancerous tumor progression. Previous studies in our laboratory have established the pro-osteogenic role of Nitric oxide/cyclic-GMP/Protein kinase G (NO/cGMP/PKG) pathway *in vitro* in osteoblasts and treatment of mice *in vivo* with NO donor, nitrosyl cobinamide and the NO-independent soluble guanylate cyclase activator, cinaciguat to protect against ovariectomy and Type 1diabetes associated bone loss without side effects. One of the major mechanisms by which the NO/cGMP/PKG pathway exerts its bone anabolic function is by enhancing the Wnt/b-catenin signaling pathway. Age-related osteoporosis is caused by decreased bone remodeling due to defective osteoblast differentiation from bone marrow stromal cells (BMSCs) and increased osteoblast apoptosis. We found reduced bone volume and bone mineral density in the aged 12-month-old C57Bl6 male mice compared to them at 3 months of age. This was consistent with the gene expression studies that showed reduced osteocalcin (osteoblast marker) and increased Cathepsin K (osteoclast marker) in the tibia. Compared to 3-month-old, the tibia of 12-month-old mice expressed less PKG1, PKG1, c-Fos, Sirt1 (master regulator of oxidative stress). Osteoblast specific PKG2 transgenic mice were partly protected from age related bone loss, with increased trabecular bone volume, trabecular number and bone mineral density. The PKG2 transgenic mice showed increased osteocalcin, c-Fos and Sirt1 expression, but decreased Cathepsin K expression. Treatment of murine primary osteoblast and bone marrow stromal cells (isolated from young and aged mice) with nitrosyl cobinamide (NOCbi) or cGMP elevating Cinaciguat, enhanced both mRNA and protein expression of *SIRT*1

showing a direct regulation of Sirt1 by NO/cGMP/PKG signaling pathway. Our studies have highlighted Sirtuin 1 (*SIRT1*) as a potential target to treat aging associated bone loss. Further studies are needed to investigate the mechanism by which *SIRT1* is regulated by NO/cGMP/PKG pathway, but for now, having the ability to increase *SIRT1* expression expands the possibilities of treatment for osteoporosis and possibly other aging associated diseases.

INTRODUCTION

1.1 Osteoporosis

Osteoporosis has been called the "silent epidemic" as it progresses relatively unnoticed until painful bone fractures occur, causing economic burden, severe morbidity, and death (1-3). Osteoporosis is a complex skeletal disorder characterized by a declining bone strength, a decreasing bone mineral density (low calcium), a compromised microarchitecture (structural integrity), and an increased susceptibility to fractures (1-3). This disorder is triggered by a disruption in skeletal homeostasis where an imbalance in bone remodeling causes the ratio of differentiated osteoclasts (bone degrading cells) to increasingly exceed the number of differentiated osteoblasts (bone forming cells), thus leading to more bone resorption (bone degradation) than bone formation (2, 3). This imbalance is influenced by a number of molecular factors which include a decrease in sex-hormone production, an increased level of oxidative stress, and reduced bone-anabolic gene expression (1-7, 10-13), mainly affecting the structural integrity of cortical and trabecular bones (1-3). The two most prevalent forms of osteoporosis are post-menopausal osteoporosis (Type-I Osteoporosis) due to an estrogen deficiency, and senile osteoporosis (Type-II Osteoporosis) due to cellular senescence (aging) (2, 3). Of these two forms, senile osteoporosis is the most difficult to treat as all influencing molecular factors manifest with aging, including Type-I osteoporosis and diabetes associated osteoporosis (10, 11, 13). Furthermore, senile osteoporosis is the most common classification of bone disease affecting more than 200 million people over the age of 50 and is responsible for approximately 8.9 million fractures a year (1-3). Unfortunately, new treatments that are safe for long term use are urgently needed as current methods present serious health risks (4-6, 10-14).

1.2 Hormone Therapies

Currently, the only treatment approved by the Food and Drug Administration (FDA) is teriparatide, a parathyroid hormone replacement shown to enhance bone formation as well as bone resorption under osteoporotic conditions (4-7, 9, 10). Consequently, this drug proved to be ineffective, as beneficial effects diminish in less than two years, and prolonged use was found to develop osteosarcomas (bone tumors) in rats (4-7). Another possible method is estrogen replacement, as estrogen enhances bone formation by producing an intracellular signaling molecule nitric oxide (NO) to promote osteoblast proliferation, differentiation and survival (13-15). Sadly, estrogen replacement is no longer recommended as the risk of developing breast cancer and thromboembolism (blood clotting) outweigh any skeletal benefits (9, 10, 12-15). Encouragingly, research on estrogen replacement therapy has shed light on nitrites (nitric oxide donors) as potential therapeutics to treat osteoporosis because NO was found to increase osteoblast proliferation by increasing activation of cyclic guanosine monophosphate (cGMP) signaling (7, 8, 10-15).

1.3 Nitric Oxide Donors

Nitric oxide (NO) is generated from arginine by the nitric oxide synthase enzymes. In osteoblasts, endothelial nitric oxide synthase (eNOS) is the predominant isoform. eNOS activates soluble guanylate cyclase (sGC) to generate intracellular cyclic GMP (cGMP) from guanosine 5'-triphosphate (GTP) (10,14,15). cGMP then activates protein kinase G (PKG) leading to stimulation of ERK, Akt phosphorylation and enhanced Wnt/β-catenin signaling via increased β-catenin nuclear translocation and target gene expression (Fig.1A) (10-14, 15, 18). Wnt induces bone development by binding to the low-density lipoprotein receptor-related protein 5 (Lrp5) at the surface of the osteoblast to inhibit the formation of the β-catenin destruction

complex (10, 11, 14, 15, 18). This allows β-catenin to accumulate in the cell and translocate to the nucleus where it will bind to the transcription factors of target genes, including bone-anabolic genes that enable osteoblast proliferation and differentiation (10, 11, 14, 15, 18). Therefore, by using NO-donors to improve the production and delivery of NO, NO/cGMP/PKG signaling can potentially improve bone formation by increasing the expression of bone-anabolic genes. However, this has proven to be difficult as all current FDA-approved NO-donors require enzymatic activation to deliver NO to cGMP. The process of enzymatic activation generates reactive oxygen species (ROS) such as O_2 and H_2O_2 as a byproduct of mitochondrial biotransformation (10, 14, 16, 18) and dramatically increases levels of oxidative stress. Increase in ROS can cause severe cardiovascular and skeletal complications (10-14) which makes using NO-donors more of a risk than a benefit (10-14).

1.4 Novel cGMP Elevating Agents

Recently, researchers at the lab of Dr. Gerry Boss have developed a novel NO-donor with the potential to increase NO production and reduce oxidative stress (13-15). Nitrosyl-cobinamide (NOCbi) is a vitamin b12 derivative with a high affinity for binding to NO **(**Fig.1B) (13, 14). However, unlike other NO-donors, NOCbi can easily release NO in the presence of NO scavengers and in cell culture media without the use of enzymatic activation (13,14). This allows NOCbi to increase cGMP signaling by delivering NO directly without generating ROS (13,14). Furthermore, the cobinamide group of NO-Cbi functions as a potent antioxidant and reduces oxidative stress by binding to and neutralizing ROS without any adverse effects presently observed (13,14,16). The lab is also investigating the effectiveness of NO-independent soluble guanylate cyclase activator, cinaciguat as a potential therapeutic approach to treat enhance PKG signaling (10). Cinaciguat is a novel cGMP elevating agent but is not a NO-donor. Cinaciguat

increases cGMP signaling by the reduction of oxidized sGC (Fig. 1C). Oxidized sGC remains inactive as it is unable to bind to NO and therefore cannot convert GTP into activated cGMP as mentioned earlier (10, 14, 15). Cinaciguat is able to reduce oxidized sGC and thus induces cGMP production, which will lead to induction of cGMP/PKG signaling and enhance bone formation (10, 14, 15). Thus far, both NOCbi and Cinaciguat have proven to be safe and effective therapeutic methods for treating mice with post-menopausal and diabetic associated osteoporosis (10-14), but as of yet, no link has been made to demonstrate their effectiveness against osteoporosis caused by cellular aging (type II osteoporosis).

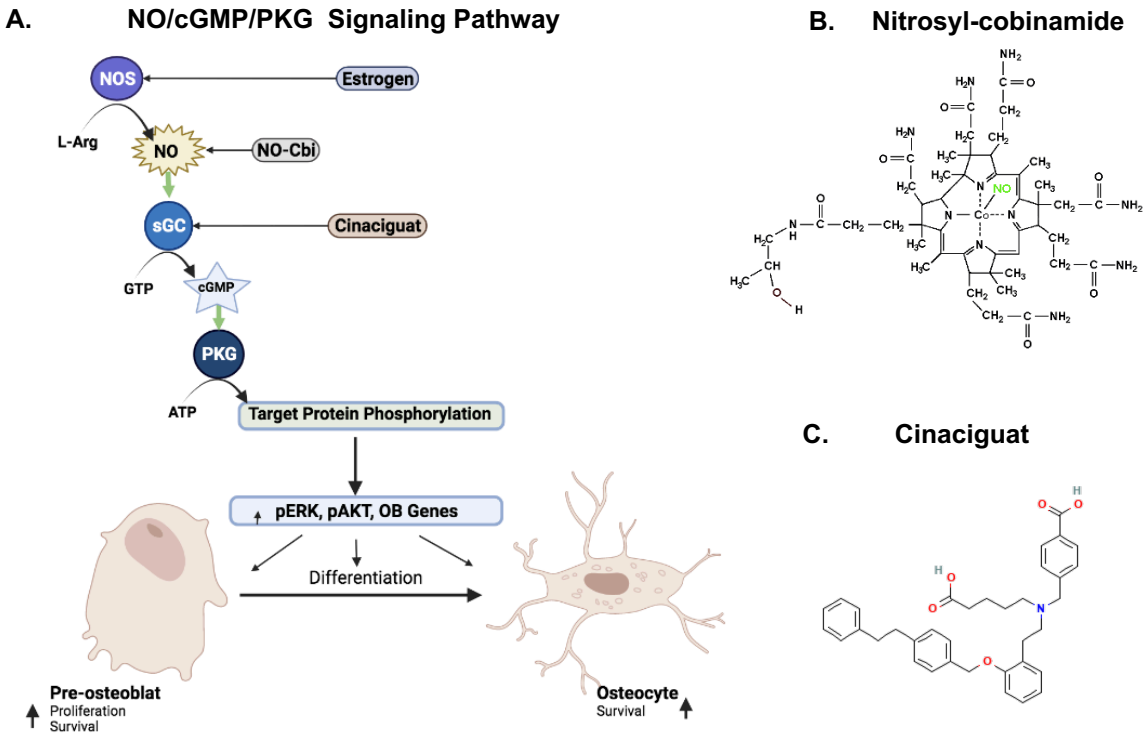

Figure 1. cGMP signaling and molecular structures of NO-Cbi and cinaciguat. (A) Pro-osteogenic function of NO/cGMP/PKG signaling. (B) Molecular structure of nitrosyl-cobinamide (cobinamide bound to NO at center carbon). (C) Molecular structure of cinaciguat.

1.5 Sirtuin1

Aging is associated with increased oxidative stress and reduced expression of several antioxidant genes involved in the glutathione and thioredoxin system, and *SIRT1* is one of the master regulators of antioxidant gene expression (17, 18). Sirtuin1 (SIRT1) is a protein deacetylase that regulates cellular senescence through the maintenance of telomere elongation (17,18). As one gets older, levels of SIRT1 decline causing telomeres to shorten (17, 18). Low expression of SIRT1 is thus associated with various aging-related diseases such as cancer, diabetes, Alzheimer's, and osteoporosis (17, 18). In previous studies, Stegen *et al. (2018)* found that SIRT1 was able to promote bone formation through the deacetylation and inhibition of sclerostin (*SOST*), which is another bone regulating gene (18). Sclerostin inhibits the Wnt/β-catenin pathway by binding to the Lrp5 receptor and blocking Wnt from deactivating the β-catenin destruction complex (18). This reduces intracellular β-catenin and prevents its nuclear translocation to express osteoblast proliferation (17,18). Additionally, sclerostin has also been found to inhibit bone formation by enhancing osteoclast differentiation through increased production of Receptor activator of nuclear factor kappa-B ligand (RANKL) (17,18). RANKL is an apoptosis regulating gene produced by dying osteoblast to recruit osteoclast resorption (1-3, 10-14, 17, 18). Thus, by inhibiting SOST expression, SIRT1 is able to promote osteoblast proliferation and bone formation while reducing osteoclast differentiation and resorption (18). While the purpose of SIRT1s role in the Wnt pathway has been well established, no studies have been conducted to analyze whether NO/cGMP/PKG signaling pathway may regulate SIRT1 and to establish whether cGMP elevating agents may be useful to treat aging associated osteoporosis.

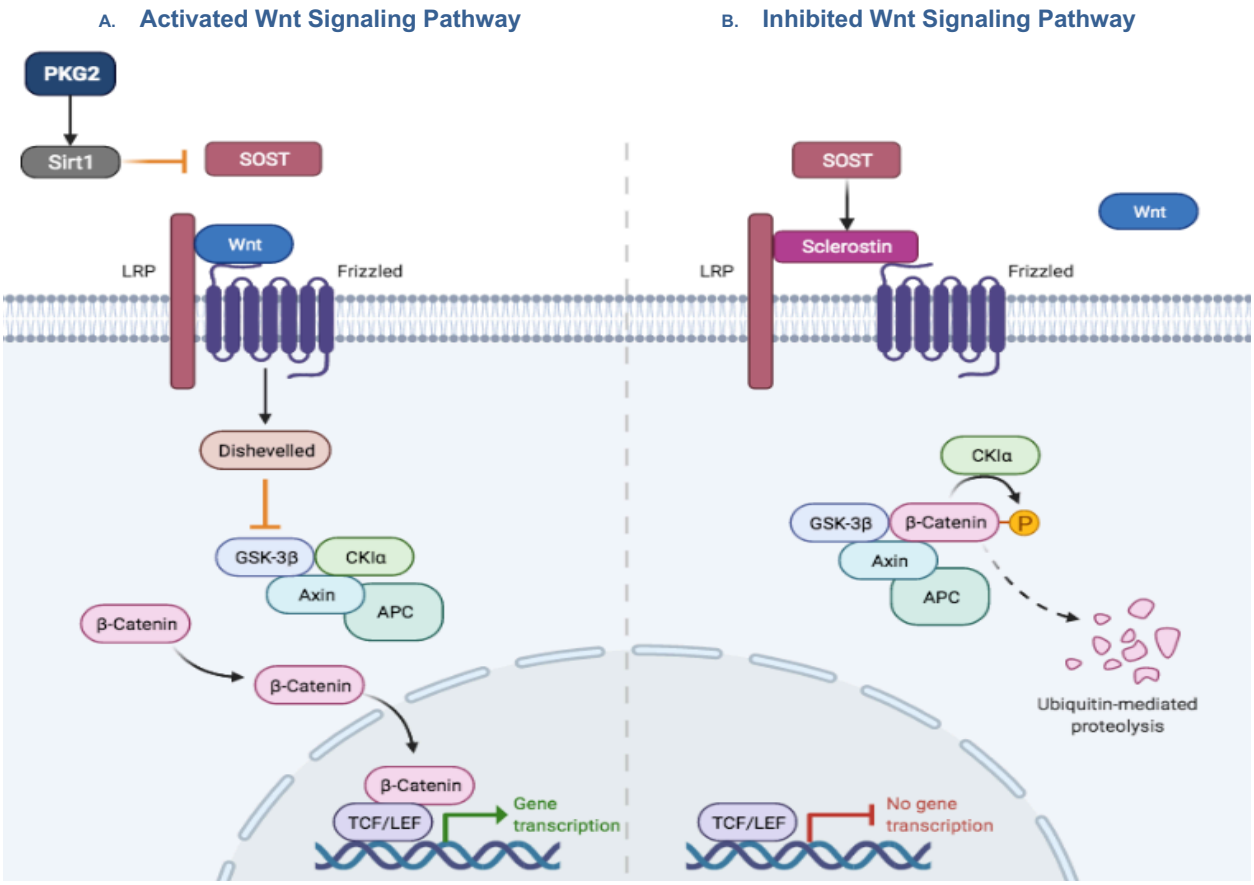

Figure 2. Evidence that Sirt1 may be downstream of PKG2 signaling. (A-B) Activated and Inhibited Wnt signaling pathway showing Sirt1 and SOST regulation of osteogenic gene transcription via β-Catenin nuclear translocation.

1.6 Central Hypothesis

Previous studies in our laboratory have established the pro-osteogenic, pro-proliferative and antiapoptotic function of the NO/cGMP/PKG signaling *in vitro* in osteoblasts. Further treatment with NO-independent soluble guanylate cyclase activator, cinaciguat protects against Type 1 diabetic bone loss (10-14). The diabetic bone disease shares several features with aging associated osteoporosis including impaired NO/cGMP/PKG signaling, increased oxidative stress as evidenced by reduced Sirt1 expression in the aging bone and reduced bone quality and microarchitecture. We hypothesize that NO/cGMP/PKG signaling pathway may regulate Sirt1 expression in the aging osteoblasts and treatment with nitrosyl cobinamide (NO-Cbi) or cinaciguat may restore Sirt1 expression in the aging bone and thus improve bone formation and protect against cellular senescence in mice with senile osteoporosis.

Accordingly, we conducted *in vitro* studies using bone marrow stromal cells isolated from young (3months old equal to 20 year old human) and aged (12 months old equal to 50 year old human) mice, treated with cGMP elevating agents Cinaciguat or NO-Cbi to measure SIRT1 expression, osteogenic differentiation and analyze oxidative stress using 8-hydroxydeoxyguanosine staining. Importantly, cGMP-elevating agents are well tolerated, indicating no adverse effects in mice treated with cGMP elevating agents and show promising activity in clinical trials for cardiovascular and neurodegenerative diseases (10-14). To establish a protective role of PKG2 in aging associated bone loss, we performed micro-CT analysis in the aged wild type and osteoblast specific PKG2 transgenic mice. The proposed studies will lay the foundation for the development of cGMP-elevating drugs as novel bone-anabolic agents and may have broader implications for pharmacological modulation of aging.

MATERIALS AND METHODS

2.1 Materials

Antibodies against SirT1 (#9475S), and normal goat serum (#5425S), were from Cell Signaling Technology. Antibodies against 8-Hydroxy deoxyguanosine (8-OHdG #GTX41980) was from Gene Tex. β-actin antibody were from Santa Cruz Biotechnology. Secondary Antibodies against Peroxidase AffiniPure Goat Anti-Rabbit IgG (#AB_2307391) was from Jackson ImmunoResearch Laboratories INC. PowerUp SYBR Green Master Mix (#A25778) from Applied Biosystems.

2.2 cGMP Elevating Agents

Nitrosyl-Cobinamide (NO-Cbi) was generated reducing dinitrocobinamide under deoxygenated conditions. Ascorbic acid and dinitrocobinamide were incubated under argon at a ratio of 5:1 for 1hr at RT; then the solution was purged with argon to remove any free NO. NO-Cbi was stable at RT for at least 1 month when filter-sterilized and stored under argon protected from light. Cinaciguat (BAY 56–2667) was purchased from Adipogen, dissolved in dimethyl sulfoxide (DMSO) and stored in aliquots at −20°C; The cGMP agonist 8-(4-chlorophenylthio)-cGMP (8-pCPT-cGMP) was from BioLog.

2.3 Generation of Col1a1-Prkg2^{RQ} Transgenic Mice

Osteoblast specific PKG2 transgenic mice Col1a1-Prkg2^{RQ}, with PKG2 under the control of a 2.3 kb segment of the mouse type I a collagen (Col1a1) promoter has been described previously (Ramdani *et al.* 2018). An N-terminal epitope tag was added to avoid problems with

enzyme mis-localization (Vaandrager *et al.* 1996), and we found that C-terminal tags reduce kinase activity. Presence of the transgene in offspring was detected by PCR analysis of tail DNA using the primers 5′CGAGCCGAAAGAGTCTACA3′ (F1) and 5′GTCTTGAAGTCCTCGCGCTCATGG3′ (R1). Mice hemizygous for the Col1a1-prkg2^{RQ} transgene were bred with wild type C57BL/6 mice from Harlan (Cumberland, IN).

2.4 Animal Experiments

10-week-old C57Bl/6 male mice were purchased from Harlan (Envigo). They were maintained in accordance with the "Guide for the Care and Use of Laboratory Animals" (2011, 8th ed., Washington, D.C., Natl. Research Council, Natl. Academies Press), and all experiments were approved by the Institutional Animal Care and Use Committee of the University of California, San Diego. Mice were housed at 3–4 animals per cage in a temperature-controlled environment with a 12-hour light/dark cycle and *ad libitum* access to water and food (Teklad Rodent Diet #8604). Wild type C57BL6 or PKG2 transgenic mice were sacrificed at 3 months (young) and 15 months (aged) of age. Femoral and tibial bones were dissected for quantitative RT-PCR, histology, and micro-CT analyses. Bone marrow stromal cells (BMSC) were isolated from young and aged mice and used for osteogenic differentiation studies.

2.5 Cell Culture and Osteoblast Differentiation

Primary osteoblasts (POBs) were isolated from tibiae of 8–12-week-old C57/Bl6 mice. Briefly the diaphysis was removed, and the marrow was flushed out, bone explants were plated for isolation of osteoblasts. Cells were cultured in 10% FBS-containing DMEM with 100-U/mL penicillin G and 100-µg/mL streptomycin. All POBs were used at passages 1–5 and characterized after differentiation in medium supplemented with ascorbate (0.3 mmol/L) and β-

glycerolphosphate (10 mmol/L), as previously described (24). Primary bone marrow stromal cells were collected by flushing bone marrow from murine femoral and tibial bones. Red blood cells were lysed in 155mM NH_4Cl, 12mM $NaHCO_3$, and 0.1mM EDTA, and the remaining cells were plated at 1.5×10^6cells/well in 12-well dishes in RPMI 1640 medium containing 10% FBS and 10% horse serum with 100-U/mL penicillin G and 100-µg/mL streptomycin. After 7 days, when the adherent cells were still at low density, the medium was switched to α-minimal essential medium supplemented with 50-µg/mL L-ascorbic acid and 5mM β-glycerophosphate to induce differentiation, and cinaciguat or 8-pCPT-cGMP was added as indicated. Medium was changed every other day with addition of drugs for 14 days. Two weeks later, colonies were stained for ALP activity, and the stained area per well was quantified by ImageJ software.

2.6 Gene expression analysis

After removing the diaphysis and bone marrow, bone shafts were snap-frozen and pulverized. RNA was purified with Trizol reagent as described previously. Quantitative RT-PCR was performed with 300 ng of RNA as described (Kalyanaraman *et al.* 2017). All primers (Supplemental Table 1) were tested with serial cDNA dilutions. Fold change in the gene of interest was normalized to *18S* rRNA. The mean ΔCT obtained for control wild type mice was used to calculate the fold change in mRNA expression in the transgenic group using the ΔΔCT method. HPRT expression was used as an internal control.

2.7 Western blotting and Immunohistochemical Staining

Primary osteoblasts were treated with 100 µM 8-pCPT-cGMP for the indicated time. Protein extracts were prepared using lysis buffer containing 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 2 mM Na_3VO_4,

10 mM NaF, and protease inhibitor cocktail. The samples were centrifuged at 13,000 g for 15 min at 4°C. Supernatants were boiled in SDS sample buffer, and proteins were resolved by SDS-PAGE and analyzed by Western blotting using anti-Sirt1 specific antibody. The densitometry analysis was performed using Image J software.

Femurs were fixed overnight in 10% formalin, decalcified in 0.5M EDTA (pH 7.5) for 5 d, and embedded in paraffin. Eight μm sections were de-paraffinized in xylene and rehydrated in graded ethanol and water. For antigen retrieval, slides were placed in 10 mM sodium citrate buffer (pH 6.0) at 80–85°C, and allowed to cool to room temperature for 30 min. Endogenous peroxidase activity was quenched in 3% H_2O_2 for 10 min. After blocking with 5% normal goat serum, slides were incubated overnight at 4°C with anti-8-OHdG antibody (1:100), followed by a horseradish peroxidase-conjugated secondary antibody. After development with 3-diaminobenzidine (Vector Laboratories), slides were counterstained with hematoxylin. Slides were scanned with a Hamamatsu Nanozoomer 2.0 HT Slide Scanning System, and image analysis was performed using the Nanozoomer Digital Pathology NDP.view2 software. Histomorphometric measurements were performed between 0.25 and 2.25 mm distal to the growth plate.

2.8 Micro-CT Analysis

Ethanol-fixed tibiae were analyzed according to established guidelines (Bouxsein *et al.* 2010), using a Skyscan 1076 (Skyscan, Belgium) scanner at 9μm voxel size, and applying an electrical potential of 50 kVp and current of 200 mA, with a 0.5-mm aluminum filter as described (Kalyanaraman *et al.* 2017). Mineral density was determined by calibration of images against 2-mm diameter hydroxyapatite rods (0.25 and 0.75 g/cm^3). Cortical bone was analyzed

by automatic contouring 3.6 to 4.5 mm distal to the proximal growth plate, using a global threshold to identify cortical bone, and eroding one pixel to eliminate partial volume effects. Trabecular bone was analyzed by automatic contouring of the proximal tibial metaphysis (0.36 to 2.1 mm distal to the growth plate), using an adaptive threshold to select the trabecular bone (Kalyanaraman *et al.* 2017).

2.9 Statistical Analyses

GraphPad Prism5 was used for two-tailed Student t-test (to compare two groups) or one-way ANOVA with Bonferroni post-test (to compare more than two groups); $p<0.05$ was considered significant.

RESULTS

3.1 Age related bone loss in C57BL/6 male mice

Computed tomography analysis (Micro CT) was performed to analyze the structural integrity and microarchitecture in the proximal tibia of C57BL/6 male mice at 3-months and 12-months of age to establish aging related bone loss (Fig.3 A-E). Our data indicated a significant decrease in trabecular bone volume (A), trabecular number (B) and bone mineral density (C) in the 12-months old while cross-sectional thickness (E) significantly increased. These results demonstrate a significant bone loss with age as older mice show significantly impaired structural integrity and bone mineral density as seen in patients with aging-associated osteoporosis (7-10).

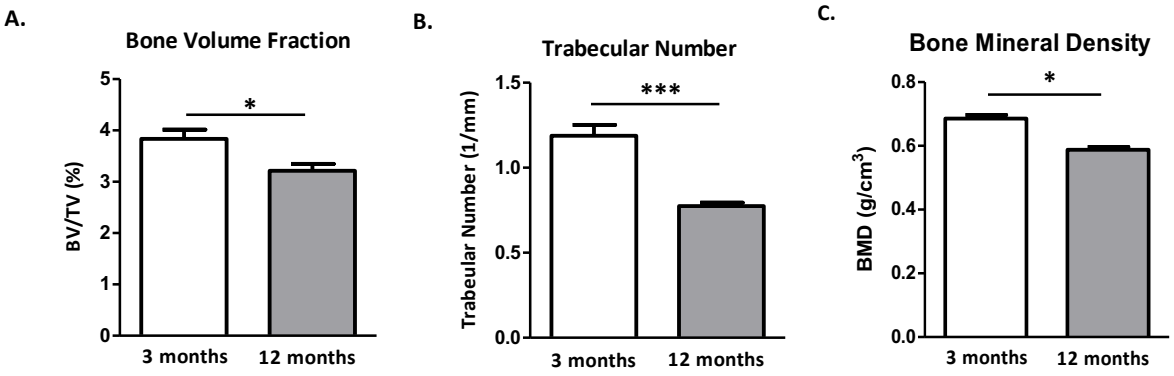

Figure 3. Age related bone loss in C57BL/6 male mice. (A-E) Micro computed tomography (Micro-CT) analysis of tibia comparing the structural integrity and microarchitecture of 3 months and 12 months old C57Bl/6 male mice. Parameters evaluated were: (A) trabecular bone volume fraction, (B) trabecular number, (C) trabecular bone mineral density, (D) cortical cross-sectional bone area and (E) cross-sectional thickness. The panel shows mean ± SEM of 5 animals/group (n=5); statistical significance was determined by two-tailed T-Test. *p< 0.05, **p < 0.01, ***p< 0.001.

3.2 Gene expression profile in the tibia of 3 months versus 12 months old wildtype mice

The tibia was dissected from 3-month and 12month old wildtype C57BL/6 male mice. The bone marrow was flushed, and the tibial shafts were pulverized to isolate RNA for gene expression analysis using qRT-PCR analysis. This was done to observe and compare the genetic disposition of bone regulating genes between young (3-month-old) and old (12-month-old) mice. The results showed a significant decline in PKG1 and PKG2 mRNA levels in the 12 months old mice compared to 3 months. This suggests an impaired NO/cGMP/PKG signaling pathway in the aged mice. Further, the expression level of the pro-osteogenic/osteoblastic marker, osteocalcin, c-Fos (PKG2 target gene) and Sirtuin 1 (master regulator of oxidative stress and antioxidant gene expression) was significantly reduced and the level of the osteoclastic gene, cathepsin (Catk) was increased in the aged mice. Previous studies in our laboratory have established a bone anabolic role of the NO/cGMP/PKG pathway and our results genetically confirmed impaired NO/cGMP/PKG pathway associated with age related bone degradation in 12 month old mice (Fig4 A-B).

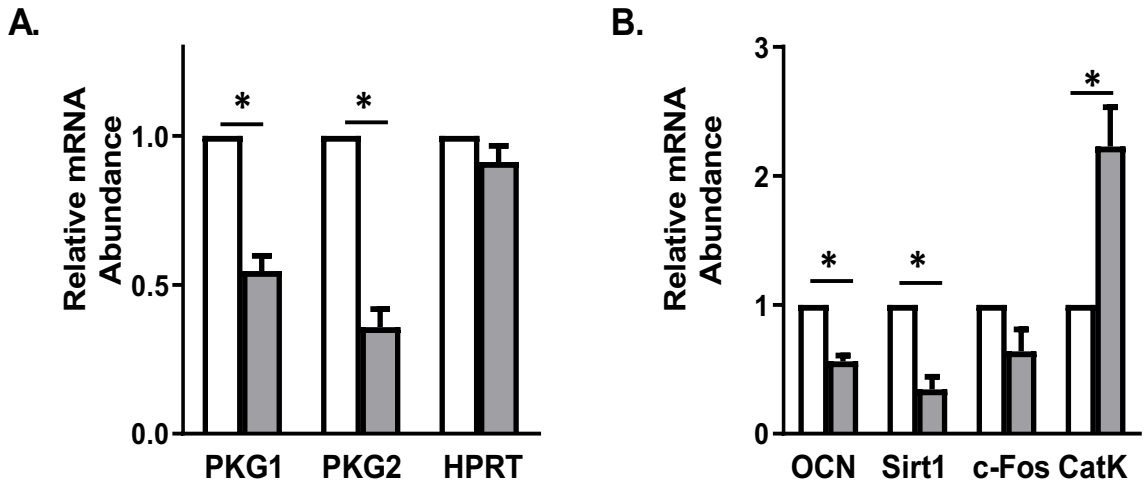

Figure 4. Gene expression profile in the tibia of 3 months versus 12 months old wildtype C57Bl6 male mice. (A-B) RNA was extracted from the tibial shafts after removal of the bone marrow and the periosteum in the 3-month and 12month old wildtype C57BL/6 male mice. The relative mRNA abundance of NO-PKG pathway/osteoblast specific genes (PKG1, PKG2, OCN, Sirt1, and c-Fos) and osteoclast specific gene (CatK) that are involved in regulating bone development was quantified by qRT-PCR analysis. Data was normalized using the housekeeping gene 18s RNA. Gene names: Protein Kinase G1 (PKG1), Protein Kinase G2 (PKG2), OCN (Osteocalcin), Sirtuin1 (Sirt1), Cathepsin K (CatK). The panel shows mean ± SEM of n=5 mice/group; Statistical significance was determined by two-tailed T-Test. *$p< 0.05$, **$p < 0.01$, ***$p< 0.001$.

3.3 Osteoblast specific PKG2 transgenic mice are partly protected from aging associated bone loss

Osteoblast specific PKG2 transgenic mice with enhanced PKG2 expression under the Col1a1 promoter were used to evaluate the role of PKG2 in aging associated bone loss. The 12-month-old Col1a1-*Prkg2*^{RQ} transgenic mice had similar tibia length compared to the 12-month-old wild type mice (Fig. 5A). The tibia of the aged wildtype C57BL/6 (WT) and PKG2 transgenic male mice (TG) were dissected and Micro CT analysis was performed to analyze/compare the structural integrity, bone microarchitecture (Fig.5 B-G) and identify differences in aging associated bone loss between mouse strains. The aged transgenic mice had higher bone volume fraction (B), trabecular number (D) and bone mineral density (E) compared to the WT controls, identifying PKG2 as a critical pro-osteogenic regulator in the process of bone development. The trabecular thickness (C) in the TG mice showed an increasing trend, however the cortical thickness (F) and Cortical TMD (G) remained unchanged between WT and TG mouse strains.

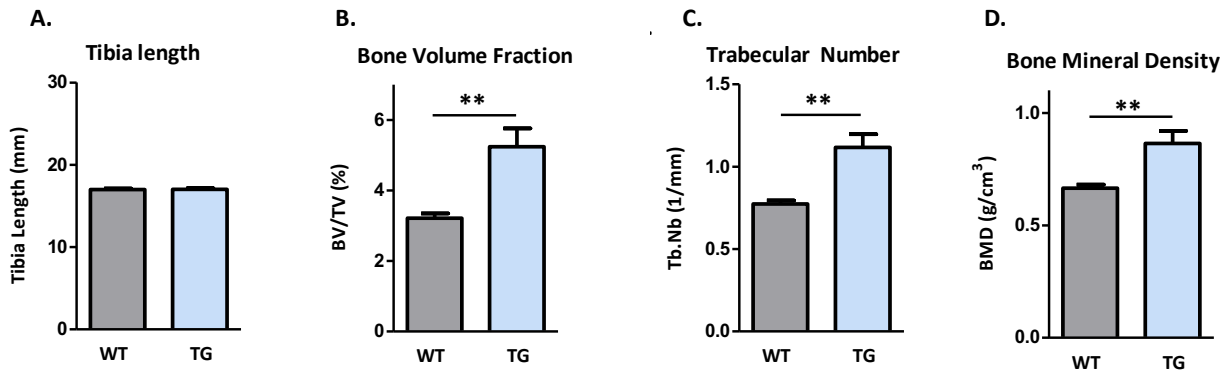

Figure 5. Osteoblast specific PKG2 transgenic mice are partly protected from aging associated bone loss. (A-G) Micro-CT analysis of murine tibia comparing the structural integrity and microarchitecture of male 12month old wildtype C57BL/6 (WT) and 12month old PKG2 transgenic mice (TG). Parameters evaluated were: (A), tibia length (B) bone volume fraction, (C) trabecular thickness, (D) trabecular number, (E) bone mineral density (F) cortical thickness and (G) cortical tissue mineral density. The panel shows mean ± SEM of n=5 animals/group; Statistical significance was determined by two-tailed T-Test. *p< 0.05, **p < 0.01, ***p< 0.001.

3.4 Gene expression profile and oxidative damage in the tibias of wildtype versus PKG2 transgenic mice

Gene expression analysis comparing tibia mRNA from 12month old wildtype C57BL/6 and PKG2 transgenic male mice was performed using qRT-PCR analysis. This was done to demonstrate the downstream regulating effect of PKG2 upregulation on osteoblast/osteoclast biomarkers OCN, Sirt1, CatK, and c-Fos (Fig.6 A). A significant higher expression of OCN, Sirt1 and c-Fos mRNA was observed in the tibia of 12month old TG mice in comparison to WT mice. Since Sirt1 plays a critical role in redox homeostasis and regulates several antioxidant genes involved in the glutathione and thioredoxin antioxidant system, we sought to determine if increased oxidative damage may contribute to aging associated bone loss. The immunohistochemistry data showed increased 8-hydroxy deoxyguanosine (8-OHdG) staining in the tibia sections of 12month old WT mice compared to young 3month old WT mice. Interestingly, the number of 8-OHdG positive cells were significantly reduced in the tibial sections of 12 month old TG mice and the levels were similar to the young 3month old (Fig.6 B-C).

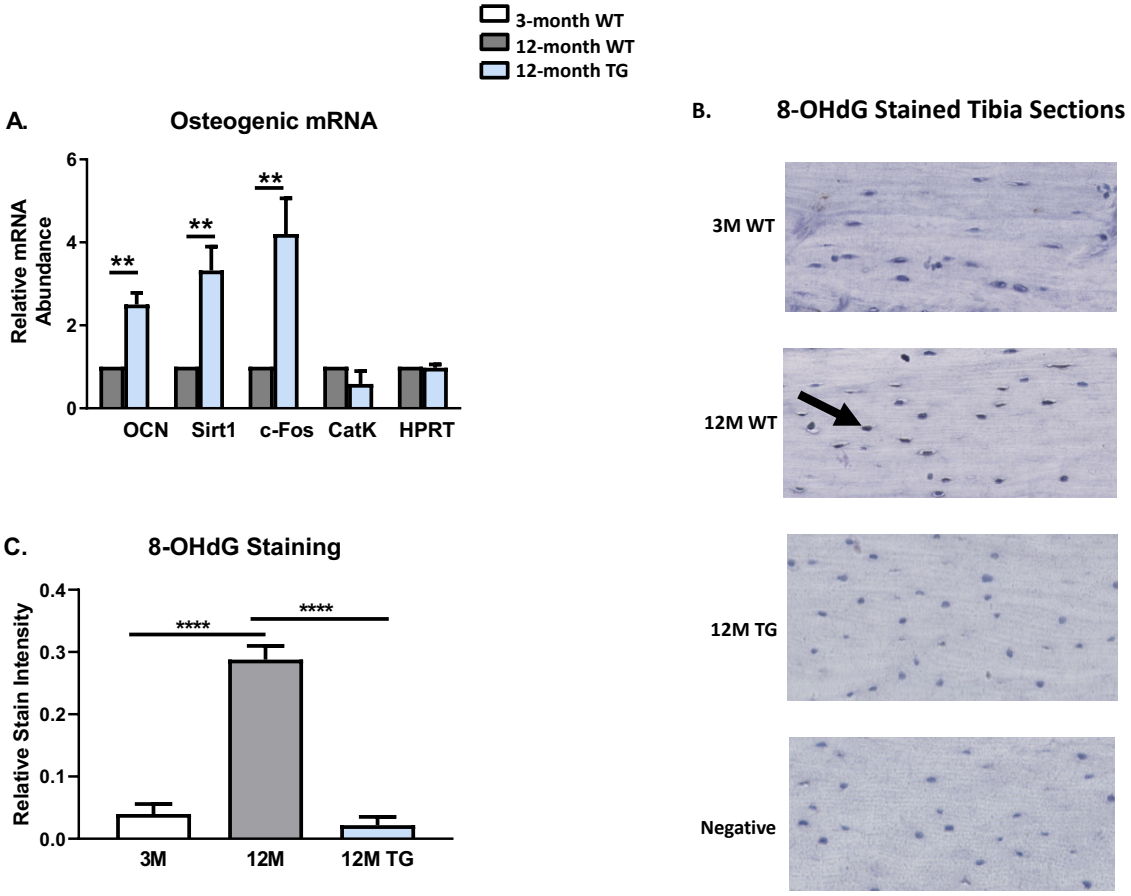

Figure 6. Gene expression profile and oxidative damage in the tibias of wildtype versus PKG2 transgenic mice. (A) RNA was extracted from the tibial shafts after removal of the bone marrow in the 12month old wildtype C57BL/6 (WT) and PKG2 transgenic (TG) male mice. The relative mRNA abundance of osteoblast specific genes (OCN, c-Fos), anti-aging gene (Sirt1) and osteoclast specific gene (CatK) that are involved in regulating bone development was quantified by qRT-PCR analysis. Data was normalized using the housekeeping gene 18s RNA. Gene names: OCN (Osteocalcin), Sirtuin1 (Sirt1), Cathepsin K (CatK). The panel shows mean ± SEM of n=5 mice/group; (B) Paraffin embedded, decalcified tibia sections of 3-month, 12-month WT and 12month old PKG2 TG mice were processed by immunohistochemical staining with an 8-hydroxydeoxyguanosine specific antibody (black) to assess osteocyte oxidative DNA damage. The percentage of damaged cells was quantified by counting the number of black cells over the number of total cells (black + blue) (C). The panel shows mean ± SEM of n=5 animals/group; Statistical significance was determined by two-tailed T-Test. *p< 0.05, **p < 0.01, ***p< 0.001.

3.5 PKG2 transgenic mice are protected from age related changes in BMSC osteogenic differentiation

Bone marrow stromal cells (BMSC) were isolated from 3 months, 12 months old control mice (WT) and 12 months old Col1a1-*Prkg2*^{RQ} transgenic mice (TG). The osteogenic differentiation and mineralization potential were analyzed using ALP and Alizarin red staining (Fig. 7A) and the ALP staining intensity was quantified using ImageJ (Fig. 7B). As reported by others, our data indicated that the aged WT mice had reduced osteogenic differentiation and mineralization compared to the young mice. Further, the BMSC from the PKG2 transgenic mice had a significantly higher osteogenic differentiation and mineralization potential (comparable to the 3-month-old) indicating a crucial role of PKG2 in the BMSC osteogenic lineage commitment and helps in restoring the impaired osteogenic differentiation in the aged mice.

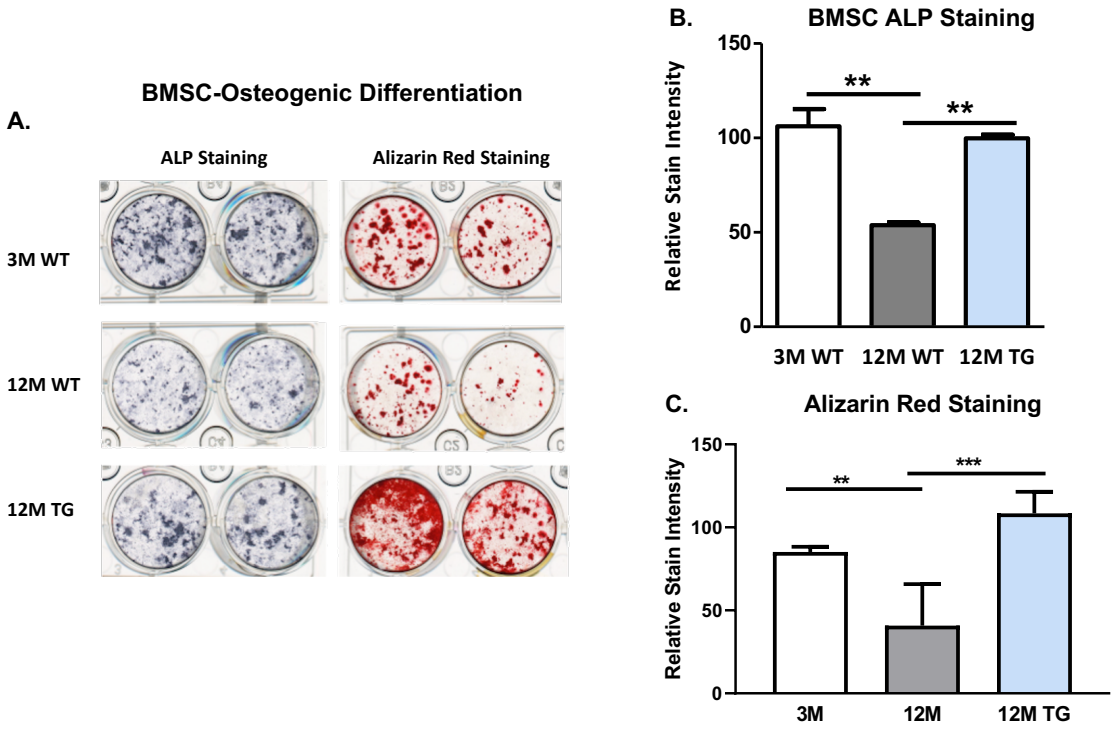

Figure 7. PKG2 transgenic mice are protected from age related changes in BMSC osteogenic differentiation. BMSCs were isolated from 3 months, 12 months old control mice (WT) and 12 months old Col1a1-*Prkg2*^{RQ} transgenic mice (TG), cultured to confluence then transferred to osteoblastic differentiation medium containing ascorbic acid, b-glycerophosphate and dexamethasone for 14 or 21 days and were stained for ALP activity or mineralization (Alizarin Red), respectively (A). The ALP staining intensity was quantified using Image J analysis (B). Data are the mean ± SEM of three to five independent experiments with n=5 animals/group. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ for the indicated comparisons by ANOVA.

3.6 NO/cGMP/PKG signaling pathway enhances Sirt1 mRNA and protein levels in murine primary osteoblasts

In order to determine whether NO/cGMP/PKG pathway regulates Sirt1 mRNA and protein expression, murine primary osteoblasts were isolated from 8week old male C57Bl6 mice and were treated *in vitro* with 8-pCPT-cGMP at various time points. Our data indicated a progressive increase in Sirt1 mRNA levels following cGMP treatment with a significant increase observed at the 6-hour time point ($p < 0.001$) compared to untreated cells (Fig. 8A). A similar kinetics was observed in the Sirt1 protein levels following cGMP treatment (Fig. 8B). The findings show that cGMP has an up-regulating effect on SIRT1 mRNA and protein expression in murine primary osteoblasts.

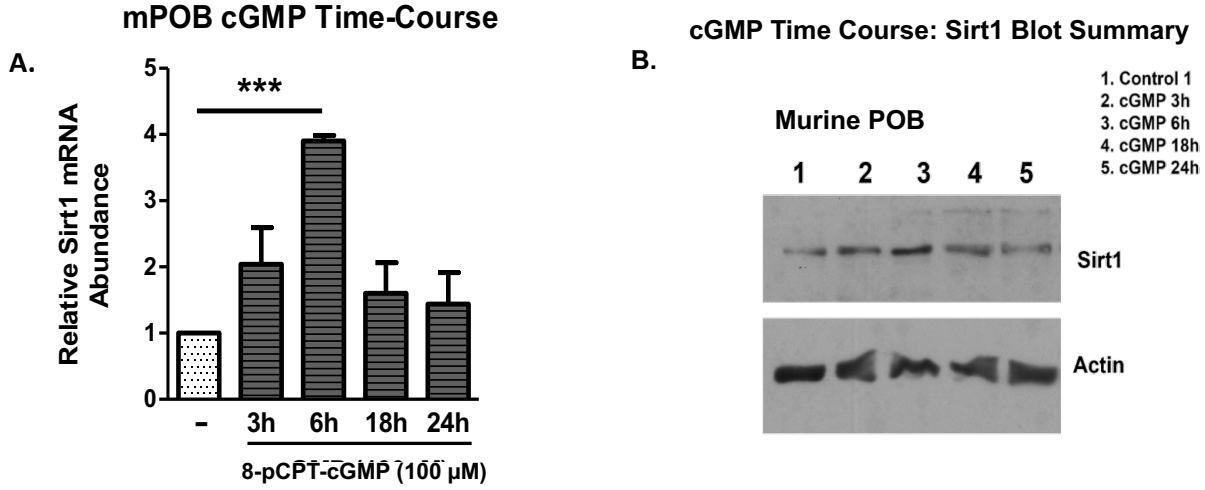

Figure 8. NO/cGMP/PKG signaling pathway enhances Sirt1 mRNA and protein levels in murine primary osteoblasts. (A) Murine primary osteoblasts (mPOBs) were isolated from 8week old male C57Bl6 mice and treated *in vitro* with 100 µM of cyclic guanosine monophosphate (8-pCPT-cGMP) for 3, 6 18 and 24 hours. RNA was isolated using Trizol reagent and SIRT1 mRNA expression was determined using qRT-PCR analysis. The data was normalized to 18S rRNA; normalized mRNA in vehicle-treated cells were assigned a value of 1. Untreated cells were labeled as control (-) as indicated with white bar. (B) In separate experiments, cell lysates were prepared following similar cGMP time course treatment (as described in A) and western blot analysis was performed using anti-Sirt1 specific antibody. The panel shows mean ± SEM of three independent experiments (n=3); Statistical significance was determined by two-way ANOVA. *$p< 0.05$, **$p < 0.01$, ***$p< 0.001$.

3.7 In vitro treatment of BMSC from 3 months and 12 months aged male mice with Cinaciguat or NO-Cbi enhances Sirt1 and osteogenic gene expression

BMSC isolated from the young (3 months) and aged (12 months) mice were subjected to osteogenic differentiation along with treatment of cinaciguat or nitrosyl cobinamide (NO-Cbi). Gene expression studies showed that both NOCbi and Cinaciguat enhanced the Sirt1, OCN and Runx2 mRNA expression in the young mice (Fig. 9A-C). Interestingly, the drugs were effective in improving the reduced osteogenic mRNA abundance and Sirt1 expression in the aged mice.

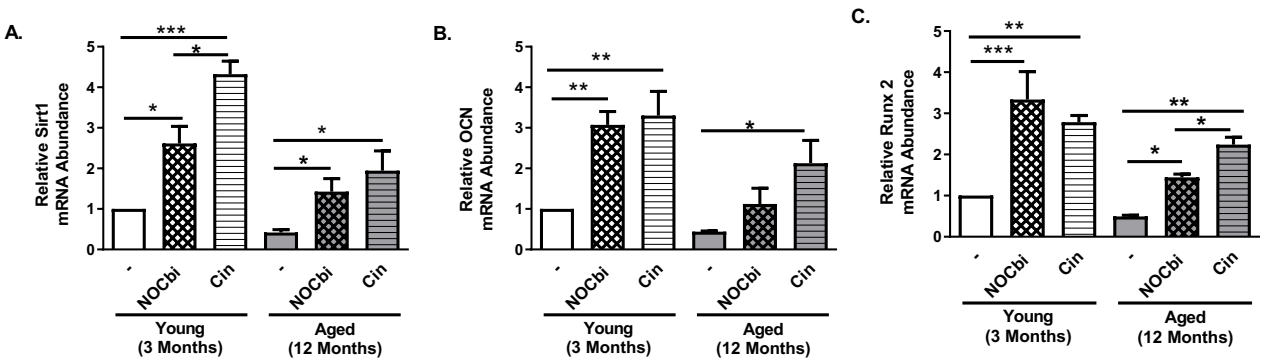

Figure 9. In vitro treatment of BMSC from 3 months and 12 months aged male mice with Cinaciguat or NO-Cbi enhances Sirt1 and osteogenic gene expression. BMSCs were isolated from 3 months (young) and 12 months old (aged) C57Bl6 male mice, cultured to confluence then transferred to osteoblastic differentiation medium containing ascorbic acid, b-glycerophosphate for 14 days along with in vitro treatment of cinaciguat (100 nM) and NOCbi (10 mM) (media change every other day with fresh drugs). Gene expression of Sirt1, OCN and Runx2 were analyzed by qRT-PCR analysis (A-C). The panel shows mean ± SEM of five independent experiments (n=5); Statistical significance was determined by two-tailed T-Test. *p< 0.05, **p < 0.01, ***p< 0.001.

DISCUSSION

To establish a basis of aging associated bone loss in wildtype C57BL/6 male mice, a micro-CT examination was performed comparing the microarchitecture of murine tibias at 3 months and 12 months of age. This demonstrated a significant decrease in bone volume fraction, trabecular number, and bone mineral density in older mice, while percent cross-sectional bone area remained relatively unchanged and cross-sectional thickness notably increased (Fig.3 A-E). As predicted, this demonstrates classic age-related bone loss as older mice are no longer able to sustain normal bone development. The increase in cross-sectional thickness indicates the physical growth of the bone over time (3 months to 12 months of growth) which implies that while the bone has grown larger in size from 3months to 12 months of age, it is now producing less bone structural factors such as calcium, leading to a weakened bone infrastructure.

These results were further supported by gene expression analysis showing a significant decrease in the abundance of osteogenic mRNA collected from mice at 12months versus 3months old, while housekeeping gene HPRT remained unchanged and served as an internal control (Fig.4 A-B). Previous studies in our laboratory established a pro-osteogenic, pro-proliferative and anti-apoptotic effect of the NO/cGMP/PKG pathway on osteoblasts development and possible regulation of sirtuin 1 (Sirt1: a master regulator of oxidative stress) expression (8-11). As expected, PKG1 and PKG2 gene expression was significantly reduced in aged mice along with Sirt1 suggesting an impairment in NO/cGMP/PKG signaling. Osteocalcin (OCN) and cellular oncogene (C-Fos) are genes involved in regulating osteoblast differentiation and mineralization while in contrast, cathepsin (CatK) is an osteoclastic biomarker involved in bone resorption. Accordingly, OCN and C-Fos were also greatly decreased in older mice while CatK was almost 2.5 times higher. Ultimately, the evidence of an impaired signaling system, a

lowered osteogenic gene expression and an increased osteoclastic gene activity, signify a greater level of bone degradation than bone formation consistent with Micro-CT analysis showing a diminished structural integrity in aging mice.

To demonstrate the specific role of PKG2 in aging associated bone loss, Micro-CT analysis was again performed on the tibias of 12 month old osteoblast specific Col1a1-*Prkg2*^{RQ} transgenic mice (TG) expressing increased levels of PKG2 (at 2.5-fold higher) under the Col1a1 promoter and compared to the tibias of wildtype (WT) C57BL/6 mice (Fig.5 A-G). Remarkably, this resulted in a substantially higher bone volume fraction, trabecular number and bone mineral density in PKG2 TG mice compared to the WT controls, indicating that the PKG2 transgenic mice are protected from aging associated deterioration in bone quality. Moreover, the tibia lengths remained the same between mouse strains indicating no effect of the transgene expression on the chondrocytes and other bone cells. Our results showed that the 12month aged transgenic mice had improved trabecular bone but no change in the cortical bone parameters such as cortical thickness and cortical tissue mineral density (TMD). Previous research has demonstrated that PKG2 mediates osteoblast proliferation effects of insulin, mechanical stimulation and pro-survival effects of estrogen by increasing ERK/Akt phosphorylation leading to enhanced Wnt/b-catenin signaling (11, 13). PKG2 transgenic mice are also protected from Type 1 diabetes associated bone loss. These findings suggest that increasing PKG2 activation through increased cGMP signaling in aging bones could improve bone mineral density and overall quality throughout the aging process.

As expected, gene expression analysis of osteogenic mRNA in TG mice versus WT mice was consistent with Micro-CT results (Fig.6 A-C). OCN and c-Fos were significantly higher in the tibias of the 12month old PKG2 transgenic mice. Interestingly, the mRNA abundance of the

anti-aging gene, Sirt1 was also enhanced in the transgenic mice compared to the control. Sirt1 is a master regulator of antioxidant gene expression and increased oxidative stress has been implicated in several pathologies including Alzheimer's, cancer and metabolic syndrome. Our data indicates that increased oxidative stress (as evidenced by increased 8-OHdG staining in the osteocytes) is a possible mechanism contributing to aging associated bone loss. Results demonstrate, that over expression of PKG2 in TG mice enhances Sirt1 expression and thus protects osteocytes from oxidative damage and therefore protects against aging induced bone loss. Furthermore, osteogenic differentiation studies were performed in the BMSC isolated from the young and aged wild type and PKG2 transgenic mice exhibiting a significant decline in the differentiation and mineralization ability in the aged WT mice. Similarly, as seen with protection against oxidative stress, the PKG2 transgenic mice were also protected from the aging associated changes in the BMSC osteogenic differentiation further demonstrating the antiaging effect of increased Sirt1 expression (Fig.7 A-B).

Finally, to determine if cGMP elevating agents (NO-Cbi and Cinaciguat) regulate Sirt1 and critical osteogenic biomarkers OCN and RUNX2 (transcription factor involved in osteoblast differentiation), we treated murine primary osteoblasts and BMSC isolated from the young and aged mice following osteogenic differentiation with NO-Cbi and cinaciguat. Excitingly, both NO-Cbi and Cinaciguat proved to be effective in significantly enhancing mRNA expression of Sirt1, OCN, and RUNX2 in young and old mice.

Overall, the findings are consistent with our predictions that cGMP may be upstream of SIRT1 and have a regulating effect on SIRT1 expression. In previous studies, cGMP signaling was found to restore bone formation in mice with post-menopausal and diabetes associated osteoporosis by activating several downstream bone-anabolic gene cascades such as the Wnt/β-

catenin pathway (10-14). In studies investigating the Wnt/β-catenin pathway, SIRT1 was also found to improve bone formation by inhibiting sclerostin, a Wnt pathway inhibitor (17, 18). SIRT1 is also known to regulate cellular senescence and thus serves as a potential therapeutic target for patients suffering from senile osteoporosis due to aging (17-18). Therefore, it is possible that SIRT1 also improves bone formation by reducing the rate of cellular aging through telomere extension (17-18). This could possibly be determined by measuring the telomere lengths of osteoblasts after treatment of cGMP *in vitro* and validating improved bone formation in aged mice *in vivo*.

The mechanisms by which cGMP regulates SIRT1 expression are not yet known and is being investigated in our laboratory (10-14). Treatment with cGMP elevating agents improves bone formation by activation of several downstream bone-anabolic signaling pathways such as Wnt/β-catenin and the Src/Erk/Akt signaling cascade (10-14). Mechanisms regulating SIRT1 expression by cGMP could potentially be identified using knockout methods to single out which genes in these pathways may have a direct effect over SIRT1 (10, 13, 17, 18). As for now, cGMP elevating agents demonstrate tremendous possibilities for not just senile osteoporosis, but for all aging associated diseases that could be mitigated through SIRT1 expression (Fig. 10). Further studies are needed to better understand the complexity of SIRT1 and its control over aging related diseases such as osteoporosis.

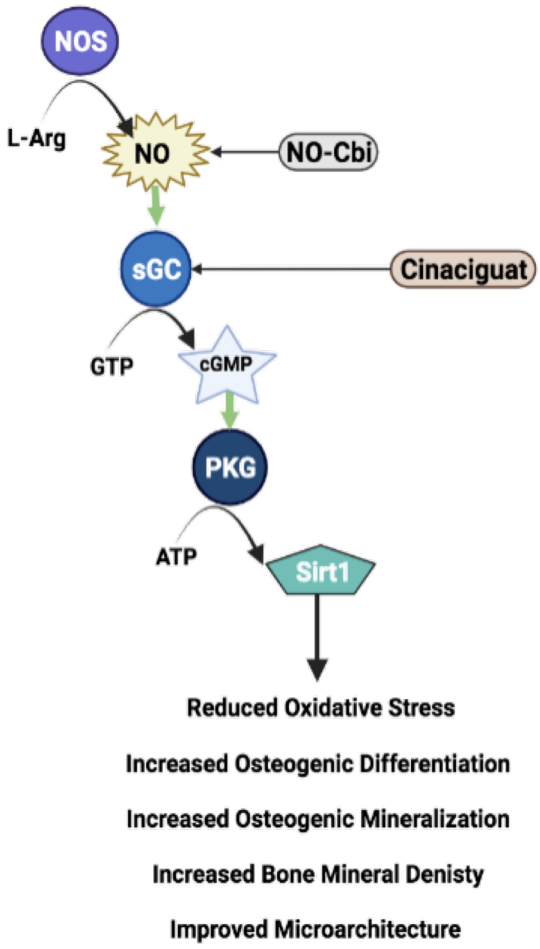

Figure 10. NO/cGMP/PKG regulating pathway of Sirt1. Purposed mechanism of PKG2 regulation of Sirt1 and its physiological benefits.

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