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Defective NO/cGMP/PKG Signaling Downregulates Sirt1 Expression in Aging-Associated Osteoporotic Mouse Model

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The Thesis of Taron Ayrapetyan is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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ABSTRACT OF THE THESIS

Defective NO/cGMP/PKG II Signaling Downregulates Sirt1 Expression in Aged Osteoblasts

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Aging associated osteoporosis is a major health concern that develops following a progressive decline in bone volume and bone mineral density with increased fracture risk. Current osteoporosis therapies like parathyroid hormone and bisphosphonate

treatment have significant side-effects like osteonecrosis of the jaw. Better understanding of the pathophysiology of age-related bone loss will be essential to identifying specific drug targets and developing novel therapeutic approaches to target osteoporosis in the elderly. Previous studies in our laboratory have shown that the nitric oxide/cyclic GMP (cGMP)/protein kinase G (PKG) pathway exerts a bone anabolic role *in vitro* in osteoblasts and osteocytes and that treatment with the cGMP elevating agent, Cinaciguat, protects against estrogen deficiency and type 1 diabetes associated osteoporosis. Our laboratory results have consistently shown reduced eNOS, PKG1 and PKG2 mRNA expression in the tibia of aged (12 month) compared to young (3 month) mice, indicating defective NO/cGMP/PKG signaling in the aged bone.

Treatment of osteoblasts *in vitro* with 8-pCPT-cGMP (100 µM) induced expression of Sirtuin 1 (Sirt 1) and its upstream transcriptional regulator, Nrf 2. We suspect that defective NO/cGMP/PKG signaling in aged bone results in the reduced Sirt1 and Nrf2 expression in the tibia of aged mice. Osteogenic differentiation studies (Alkaline phosphatase staining and gene expression analysis) from the isolated bone marrow stromal cells showed that the reduced osteogenic potential (ALP staining, and Runx2, Sirt1 gene expression) observed in the aged (wild type type) mice can be greatly improved by *in vitro* treatment with Cinaciguat and the NO-releasing agent, NO-Cobinamide. Transgenic mice with an osteoblast specific overexpression of PKG2 showed significantly increased osteogenic gene expression including Sirt1 and Nrf2 at 12 months of age. MicroCT analysis indicated that PKG2 overexpressing transgenic mice had a significant increase in bone volume, trabeculae number and bone mineral density compared to the aged (12 month) wild type mice; indicating protective

properties from the age induced bone loss. The potential regulation of Sirt1/Nrf2 gene expression by the NO/cGMP/PKG2 signaling pathway is very exciting and lays the foundation for further studies directed at understanding the mechanism of this complex regulatory system. Our results provide a novel insight into the mechanism of bone loss in aging associated osteoporosis and indicate that cGMP elevating agents should be further studied as a prospective treatment.

INTRODUCTION

The literal meaning of osteoporosis is "porous bone" and the disease is characterized by a degeneration of bone from within. But due to the honeycomb structure of healthy bone, the increased size of the pores is difficult to detect prior to fracture occurrence. Multiple systems of the body are involved in the pathogenesis of this complex skeletal disease and the molecular basis of the disease is still a mystery. It is characterized by low bone mineral density (BMD) and micro-architectural deterioration of bone tissue. As bone degrades with the erosion of calcium and collagen fibers, there is a sharp decrease in bone volume and density. Further thinning of trabeculae and cortical bone results in increased loss of bone strength and cortical porosity (Basic Biology of Skeletal Aging: Role of Stress Response Pathways, 2013.) Affected, less dense bones, become frail and more susceptible to frequent fractures and numerous associated complications.

There exist severe side effects beyond the direct consequences of broken bones that can dramatically impact the lives' of osteoporosis patients and their family members. Each year, 1.5 million osteoporotic fractures in the United States result in upwards of $15 billion in medical expense fees alone; this estimate does not account for the loss of productivity for patients and caregivers (Surgeon General, 2004). It is approximated that osteoporosis could affect more than 55% of Americans over the age of 50 and still remains one of the most under-diagnosed and under-treated disorders. The overwhelming majority of individuals will discover they are affected only when they fracture or break a bone so with increases in average life-expectancy and age-related impairment of abilities, the threat of osteoporosis also grows. It is important to elucidate

the molecular mechanism(s) contributing to the aging associated decline in bone formation and loss of bone mass to not only develop therapeutics but also hopefully discover better markers for disease progression.

The skeletal system directs a constant remodeling process of creating new bone and resorption of existing bone, while maintaining overall integrity and strength. Homeostasis is found amid the chaotic process by coordination of osteoblasts and osteoclasts, bone cells responsible for bone formation and resorption respectively. This balancing act also plays an important role in the hormonal regulation over the concentration of the bone minerals, calcium and phosphorous, in blood. As an individual ages, osteoblast numbers decrease and result in loss of bone mass and strength (O'brien, 2013). Aging rodents experience the same deterioration of mass and strength due to increased osteoblast apoptosis and reduced bone formation (Fukuda, 2002; Syed, 2010).

The stark increase in osteoporosis susceptibility in post-menopausal women has been well-documented for many years. In combination with estrogen's established bone anabolic role and the success of estrogen replacement therapies, scientists have attributed estrogen deficiency as the causal role of osteoporosis (Manolagas, 2000; Riggs, 2002). However, improved understanding of the molecular and cellular effects of aging and oxidative stress on osteoblasts and bone marrow stromal cells, combined with epidemiological evidence, is helping to elucidate the pathogenesis of age-associated osteoporosis and design more specific therapeutics. Previous studies indicate that the molecular mechanism contributing to aging associated bone loss is distinct from estrogen deficiency induced osteoporosis where increased

osteoclastogenesis is an important contributing factor (Ucer, 2016). The most devastating consequence of aging that wreaks havoc on all tissues of the body, is increased oxidative stress that has been shown to directly induce osteoblast apoptosis and inhibit osteoblast differentiation (Luo, 2004). Other deleterious effects on the skeletal system as an individual ages are thought to be in part due to the activation of cell senescence mechanisms. Senescent cells enter an irreversible low-energy state during which they are mostly dormant and can no longer divide. Senescence is important to protect tissue experiencing increased oxidative stress, telomere shortening, impaired DNA repair and nonspecific epigenetic alterations. Scientists successfully developed an osteoporotic mouse model with the aging phenotype by simply disrupting telomerase activity (in an already immunodeficient mouse) (Pignolo, 2014).

In bone tissue, senescence interrupts growth factor and intercellular signaling leading to an overall decrease of cell number and function. Aging induced senescence of mesenchymal stem cells (MSCs) is the main contributor to bone loss due the decreased bone cell division. MSCs are multipotent stromal cells with potential to differentiate into various cell types depending on their location. Those found in bone marrow in flat and long bones, differentiate into osteoblasts and eventually osteocytes. Bone integrity relies on the constant renewal and differentiation of these MSCs to produce osteoblasts and counteract osteoclast bone resorption. Once these cells enter a senescent state, they can no longer divide and osteoblast numbers begin to drastically decrease. Thus, therapeutic strategies aimed at enhancing the osteogenic differentiation of MSCs and increasing osteoblastogenesis may be an effective approach to treat aging associated osteoporosis.

Previous studies in our laboratory have established that the NO/cGMP/PKG signaling pathway (Table 2) exerts an anabolic role in osteoblasts (Kalyanaraman 2018). Results have also consistently shown decreased eNOS, NO, PKG 1 and 2 expression as individuals age and therefore decreased signaling through the NO/cGMP/PKG pathway. Further experiments showed that treatment with the cGMP elevating agent, cinaciguat, may have potential as a novel strategy for the treatment of ovariectomy induced bone loss. Endothelial nitric oxide synthase (eNOS) is the central source of nitric oxide (NO) for osteoblasts and osteocytes and so is under strict regulation (Fox, 1998). Disruption of NO production by deletion of eNOS in bone cells resulted in bone abnormalities, reduced mineralization and insensitivity to hormone treatment (Armour, 2001). At physiological concentrations, NO promotes osteoblast proliferation and activation; conversely, at abnormally increased concentrations, NO induces osteoblast apoptosis and increases osteoclast activity (Ralston, 1995). Due to its reactive nature, NO has several molecular targets. The focus of our study is on its binding and activation of soluble gaunylyl cyclase (sGC) (Montfort, 2017). Activated sGC generates cyclic GMP (cGMP) which in turn activates the soluble cGMP dependent protein kinase I (PKG I) and membrane bound cGMP dependent protein kinase II (PKG II). My work builds on previous studies done by our lab that uncovered the anabolic role of the NO/cGMP/PKG II signaling pathway in osteoblast proliferation and survival (Rangaswami, 2010). Mechanical stimulation studies using fluid shear stress have shown to induce the NO/cGMP/PKG II pathway which in turn activates osteoblast proliferation via ERK and Akt activation (Marathe 2011). Additionally, they have shown that treatment with a novel NO donor, nitrosyl-cobinamide (NO-Cbi), activated the

pathway to improve bone architecture and increase bone formation markers in both wild type and ovariectomized mice, while simultaneously decreasing osteoclast numbers (Rangaswami 2012).

Treatment with NO-generating organic nitrates has been shown to improve bone mineral density (BMD) in ovariectomized, estrogen-deficient mice (Hukkanen 2003). However, clinical application is strongly limited due to induction of oxidative stress, development of tolerance and cardiovascular complications. On the other hand, our lab has shown that the osteoprotective effects of estrogen treatment occur through nitric oxide activation of the NO/cGMP/PKG II pathway (Marathe, 2011). Additionally, treatment with NO donors and soluble guanylate cyclase activators increase PKG activity leading to increased osteoblast proliferation and survival; treatment also protects against ovariectomy and Type I Diabetes associated osteoporosis (Kalyanaraman 2017). These results suggest that the NO/cGMP/PKG II transduction pathway may be a promising target to develop therapeutics for bone loss. The aim of our study is to decipher the role of this signaling pathway in aging bone and elucidate the downstream effecter molecules contributing to aging associated osteoporosis.

Sirtuin 1(Sirt1) is a promising candidate as it is a strong regulator of cellular senescence; promoting cellular longevity by inhibiting proteins such as p53. P53 strictly monitors the integrity of the cell and detects all stress signals with several upstream and downstream signaling pathways and can direct the cell to apoptosis or senescence. Sirt1 is a member of the Sirtuin family of proteins classified as NAD+ dependent deacetylases in the nucleus, cytoplasm or mitochondria (Ott, 2018). The nuclear Sirt1

silences expression of cell cycle regulatory proteins via gene promoter histone deacetylation and heterochromatin formation; while also deacetylating numerous non-histone proteins that function as transcription factors (Fuller, 2008). Considering the osteocyte survival effects of PKG II via the pro-survival kinases ERK and Akt (Kalyanaraman, 2014) and Sirt1's role in preventing cellular senescence by activating Akt (Ouchi, 2010), we hypothesized Sirt1 to be a potential downstream target of the NO/cGMP/PKG II signaling pathway and that defective NO/cGMP/PKG signaling in the bones of aged mice leads to reduced Sirt1 expression and osteogenisis and increased cell senescence.

Due to Sirt1's activating role in various pathways controlling cell survival and proliferation, it is regulated by a complex system of negative and positive feedback loops. Following a classic negative feedback loop, increased Sirt1 protein binds a repressor complex composed of several proteins including histone deacetylases that act as a transcriptional regulator for the Sirt1 gene. In hypoxic conditions, Sirt1 protein binds a different repressor complex that again inhibits Sirt1 gene expression. This repressor complex is sensitive to redox conditions so that it disassociates during oxidative stress and allows for Sirt1 gene transcription (Ott, 2014). Sirt1 transcription is also inhibited by the important transcription factor p53 which can bind at two positions on the Sirt1 gene promoter (Ott, 2014). During starvation, the FoxO family protein, FoxO-13, will sequester p53 to allow for Sirt1 expression (Ott, 2014). The transcription factor E2F1, upregulated by genotoxic signals, activates Sirt1 transcription (Ott, 2014). In all three cases above, Sirt1 transcription is activated in response to cell stress signals to combat apoptosis and senescence. Due to the instability of Sirt1 mRNA, Sirt1 protein

levels are also regulated at the post-transcriptional level. Hu antigen R has been shown to play a critical role in stabilizing Sirt1 mRNA to increase its half-life and maintain Sirt1 protein levels. During oxidative stress, HuR is phosphorylated by Chk2 and dissociates from Sirt1; this destabilization of Sirt1 mRNA balances its increased transcription under oxidative conditions (Abdelmohsen, 2007). Post-translational mechanisms act much more rapidly to adjust Sirt1 activity in response to external stimuli. Sirt1 can localize in both the nucleus and cytoplasm, so nuclear import and export can rapidly alter Sirt1 activity (Michista, 2005). Sirt1 also undergoes post-translational modifications by the covalent attachment of small ubiquitin-related modifiers (SUMOs) at Lysine 234, which in turn recruit co-repressors to increase repression (Yang, 2007). Without the modification, Sirt1 is unable to repress p53 expression, allowing for rapid control of Sirt1 protein activity during sudden environmental changes. The upregulation of Sirt1 gene transcription during DNA damage and oxidative stress is counterbalanced by the de-sumoylation of the Sirt1 protein during these conditions (Geiss-Friedlander, 2007).

Nrf2 is a critical regulator of the endogenous anti-oxidant response system (including Sirt1) and its levels are shown to be decreased in aging tissues (Ma, 2013). The balance of oxidants and anti-oxidants maintained by Nrf2 is crucial to achieve bone homeostasis in opposing osteoblast and osteoclast activity. Animal studies knocking out Nrf2 resulted in decreased bone mass and osteoblast differentiation, while osteoclast number and activity increased (DiGirolamo, 2014). As an individual ages and Nrf2 expression decreases, the body is unable to adjust to the oxidative stress leading to an imbalance in the bone remodeling process. $Nrf2^{-/-}$ mesenchymal stem cells had reduced Sirt1 expression that could be rescued with siRNA treatment targeting p53 (JW

Lee, 2016). These results show that Nrf2 signaling preserves the stem cell nature of MSCs by inhibiting p53 and blocking its inhibition of Sirt1 to combat senescence. During normal physiological conditions, Nrf2 is localized in the cytoplasm complexed with the inhibitor molecule KEAP1 (Zhang, 2017). However, during oxidative stress, Nrf2 dissociates from its complex with KEAP1 and translocates into the nucleus. Once in the nucleus, Nrf2 binds the critical DNA repair enzyme, APE-1, and becomes activated as a transcription factor for the Sirt1 gene. Experiments with mutant APE-1 proved that its redox activity is critical for it to bind with Nrf2 in the nucleus (Yang, 2014).

To maintain bone integrity, Sirt1 directly targets a number of proteins related to bone loss such as FOXOs (regulators of anti-oxidant/anti-apoptotic genes), PGC1α/β (regulators of mitochondrial biogenesis), telomere-associated proteins, and nitric oxide synthase (NOS) (Daitoku, 2004; Yeung, 2004). Studies have shown that Sirt1 binding enhances eNOS function, activating it to increase NO levels in the cell (Mattagajasingh, 2007). Further studies showed that Sirt1 induction was blunted in eNOS-deficient mice and demonstrated a potential positive feedback loop between Sirt1 and eNOS (Nisoli, 2005). Caloric restriction studies have proven to prolong the lifespan of various laboratory organisms by enhancing their cellular Sirt1 activity (Helfand, 2004; Guarente, 2001). On the other hand, Sirt1 deletions in osteoclasts promoted their proliferation *in vitro* while NF-κB inhibition of the same cells blocked osteoclast proliferation. Sirt1 reduction also had an inhibitory effect on osteobalst differentiation which could be rescued by inhibition of NF-κB (Elefteriou, 2013). According to this data, Sirt1 regulation of NF-κB maintains bone homeostasis by controlling osteoblast and osteoclast activity. Additionally, due to the strong inflammatory effects of NF-κB, its regulation by Sirt1 may

be another mechanism to abrogate senescence and combat the detrimental effects of aging. Several studies have shown that mice over-expressing Sirt1 are protected against several age-related diseases and display increased mitochondrial activity, and decreased oxidative stress (Sanfeliu, 2017). On the other hand, mice with decreased SIRT1 expression during aging are predisposed to osteoporosis, diabetes/metabolic syndrome, and Alzheimer's disease (Tsai, 2007; Tschop, 2008).

Runx2, known as the master osteoblast transcription factor, is the first required for osteoblast lineage determination and has been shown to be under Sirt1 regulation. *Ex vivo* deletion of Sirt1 leads to decreased expression of Runx2 downstream targets, while Sirt1 agonists increased osteoblast differentiation(Guarente, 2017). Changing Sirt1 levels only affected the Runx2 downstream target expression while Runx2 levels were unchanged (Guarente, 2017). Sirt1 has also been shown to reduce resorption by inhibiting the parathyroid hormone mediated activity of matrix metalloproteinase 13 (Partridge, 2015). Another crucial cellular signaling molecule Wnt, promotes osteoblast proliferation, function and survival (Ott, 2014). Forkhead boxO (FoxO) proteins sequester Wnt and thus inhibit osteoblast proliferation and activity. Sirt1 promotes osteoblast generation by deacetylating FoxO proteins to decrease their expression and allow Wnt signaling to induce osteoblastogenesis (Almeida, 2014). Interestingly, it has been shown that besides promoting osteoblast proliferation, Wnt activity can also inhibit bone resorption by reducing osteoclastogenesis (Ohlsson, 2014).

Drug therapeutics for osteoporosis is extremely limited. A more complete understanding of its molecular mechanisms will help identify potential targets and design better diagnostic techniques. The skeletal benefits of estrogen replacement

therapies are becoming outweighed by their carcinogenic potential. Most other pharmaceuticals are designed to target the proliferation and growth of osteoclasts but their nonspecific activity can inhibit osteoblast growth as well (Reid, 2015). Since aging-induced osteoporosis results from decreased osteoblast activity, osteoclast inhibitors are a near-sighted treatment and less efficacious. Our lab has shown success in treating ovariectomy associated bone loss with the NO donor, nitrosyl cobinamide (NO-Cbi), activating the NO/cGMP/PKG signaling pathway (Kalyanaraman 2017). The only FDA-approved bone anabolic agent, recombinant PTH, can only be used for two years due to decreased efficacy and carcinogenic risk. The only FDA-approved NO donor for long term use are organic nitrates which induce oxidative stress and lose effect over time (Parker, 2004). Enzymatic release of NO from nitrates produces reactive oxygen species which have harmful effects in all tissues, including the skeletal system (Gori, 2007).

We hypothesize that the defective NO/cGMP/PKG signaling in the osteoblasts and bone marrow stromal cells (BMSCs) isolated from the aging bone may lead to reduced Sirt1 levels resulting in increased oxidative stress and cellular damage. In turn, this results in reduced osteogenic differentiation and increased osteoblast apoptosis, contributing to aging associated osteoporosis.

MATERIALS and METHODS

Osteogenic Differentiation and ALP Staining

Murine primary osteoblasts isolated from bone chips of femurs and tibiae of sacrificed C57BL6 mice 8-12 weeks were pooled and cultured in DMEM 1X (4.5g/L glutamine) media (sigma) supplement with 10% fetal bovine serum (FBS, sigma Aldrich) 100- µg /mL penicillin G, 100-µg/mL streptomycin penicillin, and fungizone. When cells reached confluence, media was supplemented with 0.3mM ascorbic acid and 10 mM β-glycerolphosphate and changed three times per week to induce differentiation. After 14 days, cells were washed with 1% PBS and fixed with 3.7% formaldehyde for 8 minutes. Following 3 washes with 1% PBS, cells were stained for 30 minutes in the dark with ALP staining solution: 1M Tris, 5M NaCl and 1M $MgCl_2$.buffered to 9.5 pH, combined with 5-Bromo- 4-Chloro- 3-Indolyl Phosphate (BCIP) dissolved in dimethylformamide and nitro blue tetrazolium chloride (NBT) dissolved in water. Plates were scanned and staining intensity was analyzed using ImageJ software.

Primary bone marrow stromal cells were collected by flushing bone marrow from the femurs and tibia of sacrificed mice. Cell suspension was treated with red blood cell lysis buffer (155mM NH_4Cl, 12mM $NaHCO_3$ and 0.1 mM EDTA) for 10 minutes at room temperature and centrifuged at 1500 rpm for 3 minutes. Buffer was discarded and the cell pellet resuspended then plated at $1.5x10^6$ cells per well in a 12-well dish with 1 mL media.

Western Blot Analysis

Following overnight starvation with DMEM media supplemented with 0.1% FBS, murine primary osteoblasts were treated with 100 μM cGMP for 3 hours, 6 hours, 18 hours, and 24 hours. Cells were lysed using 120 μL of hot 1x SDS-sample buffer and cell lysate was collected. Following centrifugation and boiling for 2 minutes, samples were sonicated at the lowest speed for 1 second. After another round of centrifugation and boiling, cell lysates were ready to be loaded on SDS-polyacrylamide gels. Protein samples were separated on 9% SDS-PAGE gels and transfer was done using Immobilon-PVDF membranes at 500mA for 2 hours. The membrane was then blocked in 5% milk in tris buffered saline containing 0.1% Tween 20 (TBS-T). Overnight primary antibody incubation using anti-Sirt1 antibody (1:1000) was followed by horseradish peroxidase conjugated secondary antibody incubation for one hour at room temperature. Enhanced chemiluminescense (Thermo) were used to generate western blots as described previously (Guidi, 1997). Blots were analyzed using ImageJ software (nih.gov).

8-Hydroxydeoxyguanosine (8-OHdG) staining of aorta paraffin sections

Aortas isolated from sacrificed mice were fixed in 10% neutral formalin solution overnight, decalcified in EDTA (pH7.5) for 5 days, and embedded in paraffin. Hydrating paraffin sections required incubating in xylene twice for 3 minutes, xylene 1:1 with 100% ethanol for 3 minutes, 100% ethanol twice for 3 minutes sections required incubation in kopplen jars with xylene, 100% ethanol (sigma) and 95% ethanol each, 95% ethanol three times, 70% three minutes and finally 50% ethanol for three minutes. Slides were washed twice in 1% PBS before antigen unmasking was performed by incubating slides in boiling 10mM citrate solution (Sigma) for 10 minutes. (Following incubations all done in humidified chamber). After washing with 1% PBS twice for two minutes, the slides were

incubated with 100µg/mL RNase A (dissolved in RNase dissolving buffer- 10mM Tris, 1mM EDTA, 0.4M NaCl) for one hour at 37°C. After washing with 1%PBS, slides were incubated for one hour at room temperature with 10µg/mL Proteinase K (ThermoFisher) dissolved in 1%PBS. Following washing with 1%PBS slides were incubated with 4N HCl for seven minutes at room temperature to denature DNA. After washing with 1%PBS sections were incubated with 0.2% Triton X 100 for fifteen minutes at room temperature. Slides were rinsed with 1%PBS and incubated in 0.5% goat serum in PBS for 1 hour at room temperature. Blocking solution was vacuumed off and slides were incubated with anti-OHdG primary antibody (GeneTex) diluted 1:50 in blocking solution overnight at 4°C. Slides were washed twice with 1%PBS and endogenous peroxidase blocked by incubating with 3% hydrogen peroxide (Sigma) for ten minutes in a coplin jar. Sections washed in autoclaved water twice for three mintues each and then once in 1% PBS for five mintues. Sections were washed in 1% PBS two times for three minutes and incubated in mouse HRP conjugated secondary antibody (CellSignaling) one hour at room temperature. Sections were washed three times for five minutes in 1% PBS and stained with DAB substrate (Vector) in humidifier for ten minutes. Slides were immersed in autoclaved water and washed in 1%PBS before being counsterstained with heamotoxylin (Sigma) for eight minutes. Slides were immersed up and down in jars of autoclaved water and 1%PBS to minimize residual staining and washed in two changes of autoclaved water for two minutes each. Slides were then dehydrated in coplin jars of 95% ethanol, 100% ethanol and finally xylene for one minute each. Slides were allowed to dry on benchtop before mounting coverslips using Fluoromount-G (SouthernBiotech).

Coverslip edges were sealed with nail polish and finally visualized with a fluorosecent microscope and analyzed using nanozoomer.

Immunofluorescence Staining and Bromodeoxyuridine staining Proliferation assay

Murine primary osteoblasts (1x10^4) plated on glass coverslips, were first starved overnight with the DMEM media containing 0.1% FBS as mentioned above. After the indicated treatment, cells were labeled with 200 μM BrdU for 18 hours. Cells were washed with 600 μL of phosphate buffered saline (PBS), fixed with 3.7% formaldehyde for 10 minutes and washed again with PBS. After cell permeabilization using 0.5% Triton X-100 for 15 minutes, DNA was digested with DNase I (Sigma) for 30 minutes at 37°C. After blocking with 2% BSA in PBS, cells were incubated with goat anti- mouse BrdU antibody (Sigma) and Hoechst 33342 primary antibodies followed by FITC labeled secondary antibody. Cells were visualized under a fluorescent microscope and analyzed as described (Rangaswami, 2010).

RNA Isolation, cDNA Synthesis and Quantitative RT-qPCR

RNA extraction was performed with TriReagent™ (Molecular Research Center, Inc.); cDNA synthesis was done using the iScript cDNA Synthesis Kit (BioRad) following manufacturer instructions. PCR parameters set to 30 s of denaturation at 95 °C, annealing for 45 s at 60 °C and 1 s extension at 72°C for 40 cycles. Quantitative RT-PCR done with MX3000 real time PCT, Stratagene detection system and IQ™ SYBR Green Supermix (Bio-Rad). 40 cycles confirmed the absence of nonspecific amplification and a single PCR product for each primer pair. mRNA expression levels were analyzed on

excel with the $2^{-\Delta\Delta Ct}$ method, using the housekeeping *18S* as a control reference to correct for differences in RNA extraction or varying reverse transcription efficiencies.

Microcomputed Tomography

The right tibia isolated from sacrificed mice was wrapped in PBS soaked kimwipes and frozen. After removal of soft tissue and fixation in 70% ethanol, Micro-CT analyses were performed using Skyscan 1076 (Kontich, Belgium) scanner at 9 µm voxel size and applying an electrical potential of 50kVp and current of 200 µA, with a 0.5 mm aluminum filter. Trabecular bone volume, bone mineral density and trabecular number were analyzed as described earlier (Kalyanaraman, 2018).

Statistical Analysis

Graph Pad Prism 5 was used for two-tailed Student t-test (to compare two groups) or one-way ANOVA with Bonferroni post-test analysis (to compare more than two groups); $p<0.05$ was considered significant.

RESULTS

Bone Marrow stromal cell (BMSC) isolation and osteogenic differentiation studies:

Bone marrow stromal cells were isolated from 3 month (young) and 12 month (aged) C57BL6 background male wild type and transgenic mice over expressing PKG2 protein (osteoblast specific). These cells were used for the osteogenic differentiation studies. The bone marrow was flushed into a plate with α-MEM containing 10% FBS and supplemented with pennicllin/streptomycin. The cell pellet was treated with RBC lysis buffer for 10 minutes at room temperature. The media was changed 48h later. Confluent cultures were cultured in differentiation media containing ascorbic acid, β-glycerophosphate and dexamethasone for 14 days and stained for alkaline phosphatase using BCIP/NBT as substrate. Increased osteogenic differentiation (ALP staining) was observed in the BMSCs isolated from the 3 month old mice compared to the ones isolated from the aged 12 month old wild type. Interestingly, increased ALP staining was observed in the BMSCs isolated from the aged (12 month) transgenic mice compared to the wild type; the osteogenic differentiation was comparable to the 3 month wildtype controls indicating a possible pro-osteogenic role for PKG2 (Figure 1).

Gene expression profile in tibia of young and aged mice

To determine the gene expression profile in the tibia of the young and aged mice, RT-qPCR was performed. Briefly, tibia (devoid of the bone marrow) was pulverized in liquid nitrogen and total RNA was isolated using Trizol reagent. Following reverse transcription, cDNA was used for PCR using Bio-Rad Sybr Green Mastermix. Aged mice showed a significant decrease in both PKG I and II mRNA levels compared to the young mice while HPRT levels were unchanged (Figure 2a). Additionally, the aged mice displayed increased expression of the osteoclast marker Cathepsin K (CatK) and decreased expression of the pro-osteoblastic protein osteocalcin (OCN) (Figure 2b). The most interesting finding that would guide the rest of my experiments was the significant decrease in the Sirt1 mRNA in aged mice (Figure 2b).

MicroCT analysis of 2 months vs 12 months Wild type C57BL6 males

To analyze the physical effects of aging on bones, we isolated the femurs of young and aged mice. MicroCT analysis focused on three important parameters of bone health: ratio of (mineralized) bone volume to total volume, trabecular number and trabecular separation. The bones of aged (12 month) mice showed a significant decrease in their BV/TV ratio compared to that of young mice (2 months) (Figure 3a). Trabecular thickness was assessed by calculating the average trabecular number and trabecular separation. As expected, the bones of aged mice had a significantly decreased trabecular number (Figure 3b) and sharp increase in trabecular separation (Figure 3c).

BMSC differentiation and gene expression analysis

Bone marrow stromal cells isolated from young and aged mice were allowed to reach confluency and cultured in differentiation media for 14 days. RNA was extracted from cells using Trizol and reverse transcribed to cDNA following the iScript protocol. RT-qPCR analysis with the cDNA was used to calculate relative mRNA abundance in the cells. Basal levels of transcription of the pro-osteogenic markers Runx2 (Figure 4a) and OCN (Figure 4b) trended downward as expected. Treatment with the soluble guanylate cyclase activator, cinaciguat, and nitric-oxide donor, NO-Cobinamide, significantly increased expression of Runx2 in both young and aged mice (Figure 4a, 4b). In agreement with current research, expression of our anti-oxidative protein Sirt1 was also significantly reduced in aged mice; treatment with cinaciguat and NOCbi significantly improved Sirt1 gene expression (Figure 4c).

Gene expression in aged WT and aged transgenic mice

To further gain a mechanistic insight into the pro-osteogenic and proliferative role of PKG2 in the aged mice, we analyzed gene expression in the bones of 12 month old wild type and transgenic mice overexpressing PKG II specifically in the osteoblast. Table 1 confirms the increased expression of PKG II in the transgenic mice only. RT-qPCR analysis reveals increased expression of the pro-osteoblast markers, OCN and cFos, in aged transgenic mice compared to aged wild-type mice (Figure 5). The important anti-aging protein Sirt1 was also transcribed more in transgenic mice compared to wild-type(Figure 5). Transgenic mice seemed to down regulate expression of the pro-

osteoclast gene CatK(Figure 5). Lastly, both wild type and transgenic mice had equivalent expression of PKG I protein (Figure 5). The results were normalized to 18S mRNA and HPRT served as an internal control.

Micro-CT analysis of aged WT mice vs aged transgenic mice

The right femur isolated from male aged wild type and PKG2 over expressing transgenic mice in the C57BL6 background were stored in 70% alcohol and used for micro-CT analysis. The transgenic mice showed a statistically significant improved ratio of bone volume to total volume compared to aged wild type mice (Figure 6a). Transgenic mice also had a significantly improved trabecular number (Figure 6c) and therefore trabecular thickness (Figure 6b) compared to the wild type mice. Bone mineral density (BMD), an important factor in bone health, was also significantly improved in the aged transgenic mice compared to the wild type controls (Figure 6d).

Western blot analysis of bone marrow stem cells treated with cinaciguat and NO-cobinamide

Murine primary osteoblasts isolated using bone chips from the tibias and femurs of sacrificed young and aged mice were used to analyze the signaling pathway downstream from PKG II. The cells were treated with vehicle, cinaciguat (100 nM) or NO-cobinamide (10 uM) then lysed and sonicated for western blot analysis using pAkt primary antibody and HRP-conjugated goat anti rabbit secondary antibody (Proteintech). Results confirmed increased basal expression of pAkt in younger cells

compared to aged cells; both aged and young cells responded positively to treatment with cinaciguat and NO-cobinamide as shown with the increased pAkt expression (Figure 7a).

Another group of the murine primary osteoblasts were treated with 100 uM 8-p CPT-cGMP at the indicated time points. Cells lysates were again used for western blot analysis using anti-Sirt1 primary antibody and HRP-conjugated goat anti mouse secondary antibody (Proteintech). Treatment with cGMP resulted in increased expression of the anti-senecense protein Sirt1, with maximal expression at the 16 hour time point (Figure 7b).

To determine the mechanism by which NO/cGMP/PKG signaling pathway activates Sirt1 expression, we analyzed the gene expression profile of its transcription factor Nrf2 after shorter cGMP treatment periods of 30 minutes, 1 hour and 2 hours. RT-qPCR data revealed a significant increase in Nrf2 expression upon treatment with cGMP for 1 and 2 hours compared to control (Figure 7c). Nrf2 mRNA levels in the tibia of WT young, untreated WT aged and transgenic mice (osteoblast specific PKG II over expressing) was analyzed using RT-qPCR. The Sirt1 activator Nrf2 is significantly down regulated in aged mice compared to young mice; and in agreement with our previous data the transgenic mice over expressing PKG 2 rescued Nrf2 expression (Figure 7d).

FIGURES

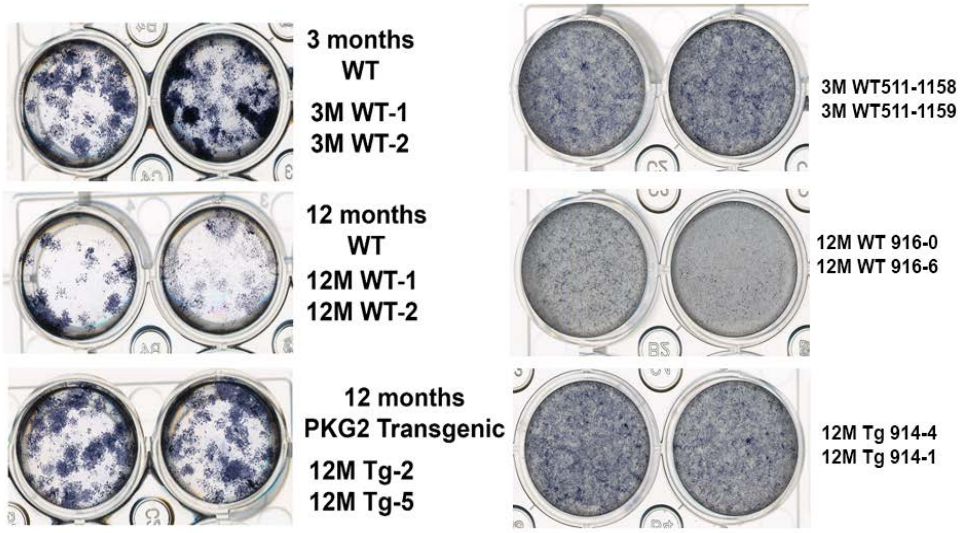


Figure 1: Bone Marrow Stem Cell Alkaline Phosphatase Staining. BMSC were isolated from young (3 month), aged (12 month) male wildtype and transgenic old mice with osteoblast specific over expression of PKG2. Isolated cells were cultured in differentiation media for 14 days and assessed for alkaline phosphatase activity with a cytochemical staining using 5-bromo-4-chloride-3-indolyl phosphate and nitro blue tetrazolium. Blue color indicates positive staining.

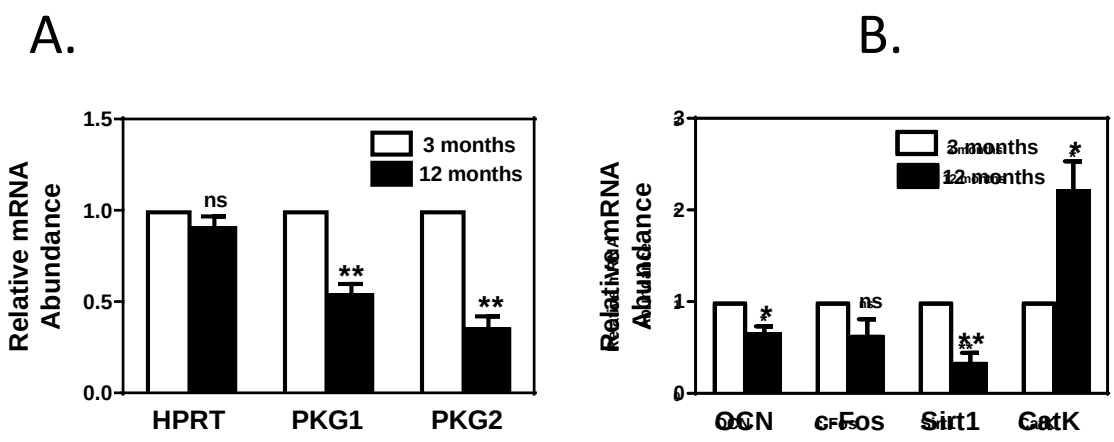


Figure 2: Gene expression profile in the tibia of young (3 month) and aged (12 month) mice. *A*, RT-qPCR analysis revealed a siginificant decrease in both PKG I and PKG II mRNA abundance in the tibia of aged mice compared to the young mice while HPRT levels were unchanged. *B,* Additionally, aged mice displayed an increased expression of cathepsin K (osteoclast marker) and decreased osteocalcin (osteoblast marker) and Sirt1expression in the tibia. RT-qPCR raw data was normalized to 18S mRNA.

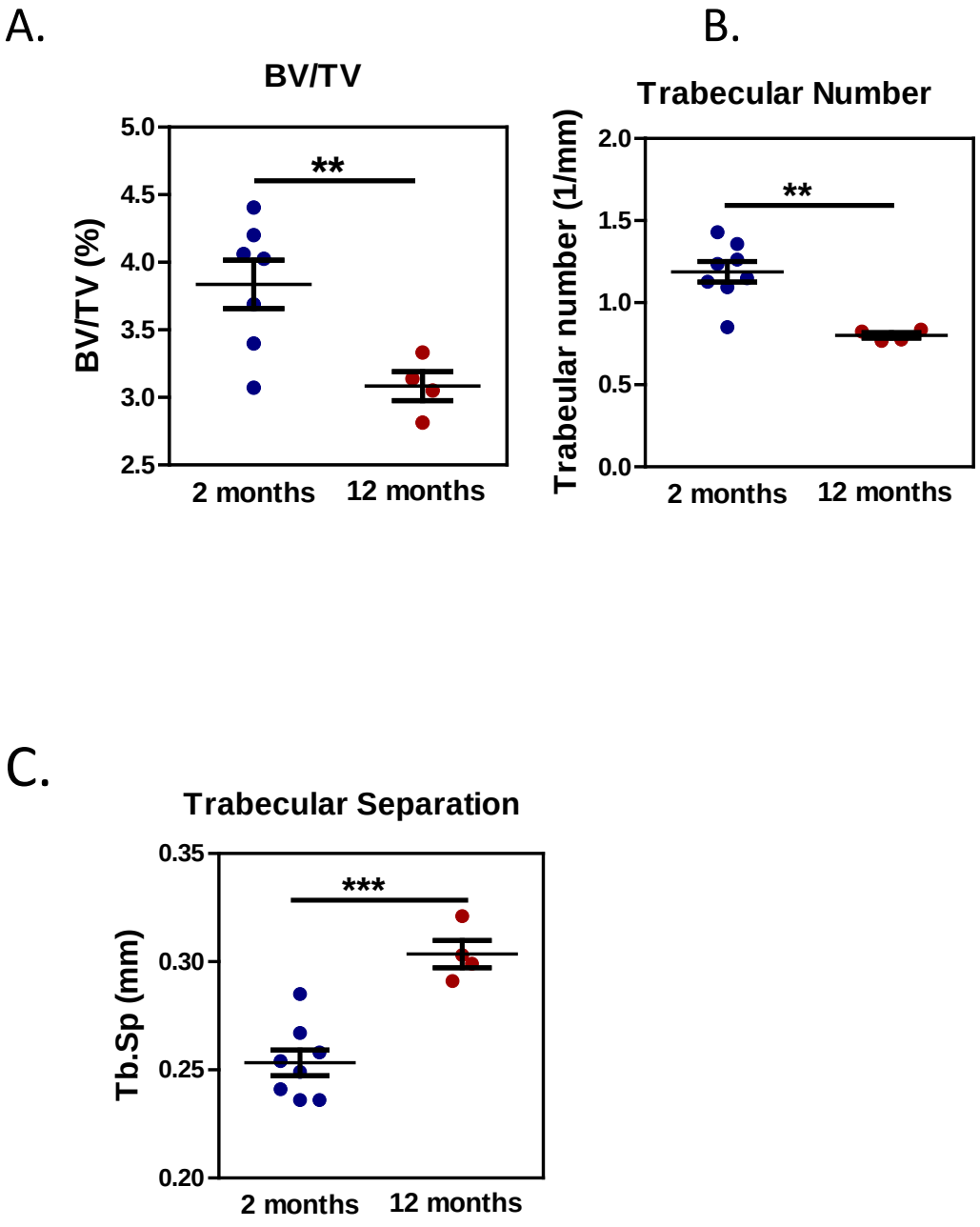
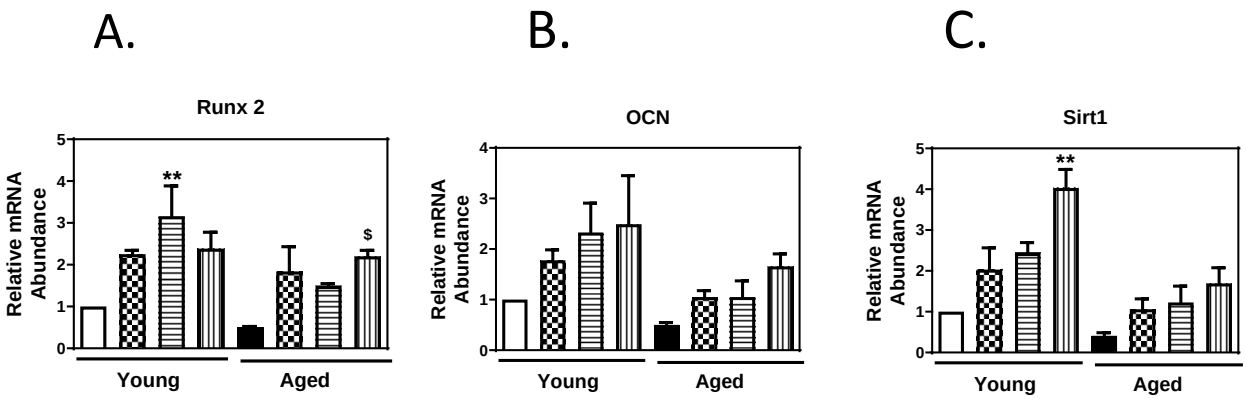


Figure 3: MicroCT- Trabecular and Cortical bone parameters 2 months vs 12 months Wild type C57BL6 males. *A*, Comparison of the ratio of bone volume to total volume in young and aged mice. *B*, Compares trabecular number in young and aged mice. *C*, Young versus old trabecular separation.



1. Young Differentiated
2. Young Diff + Cbi 10 μM
3. Young Diff + NOCbi 10 μM
4. Young Diff + Cin 100 nM
5. Aged Differentiated
6. Aged Diff + Cbi 10 μM
7. Aged Diff + NOCbi 10 μM
8. Aged Diff + Cin 100 nM

Treatment for 48h

Figure 4: Differentiated BMSC gene expression analysis. RNA extracted from differentiated BMSC was reverse transcribed to cDNA and used in RT-qPCR analysis. *A,* Basal expression of Runx2 trends downwards in aged mice; treatment with Cinaciguat and NO-Cobinamide increases Runx2 transcription in young mice. *B,* OCN gene expression is also decreased in aged mice compared to young mice. *C,* Sirt1 gene transcription again decreased in aged cells and in vitro treatment with cinaciguat and NO-Cbi greatly improved the Sirt1 mRNA levels.

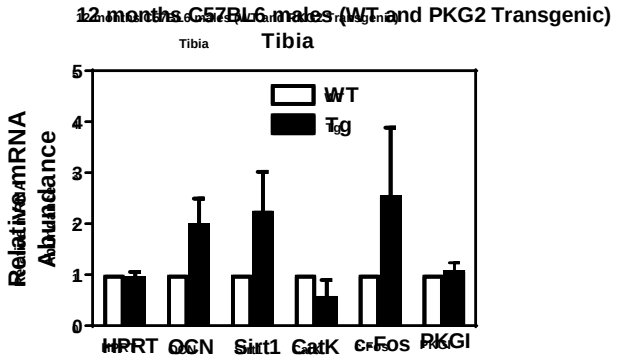
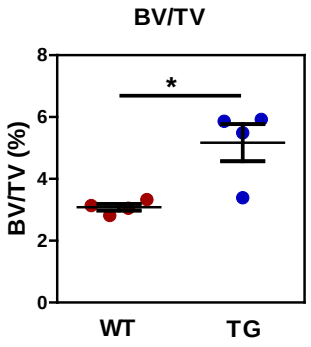


Figure 5. 12 months C57BL6 males WT and PKG2 transgenic gene expression in tibia. Increased abundance of PKG2 protein led to increased expression of pro-osteogenic markers (OCN, c-Fos) and a simultaneous decrease in the bone resorption protein Cathepsin K (CatK) as compared to expression in tibia from wild-type mouse.. PKG I levels were unchanged in transgenic mice over expressing PKG2. Abundance was normalized to 18S mRNA.

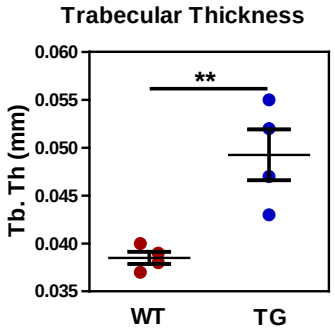
Table 2. Detection of PKG II transgene by QPCR- CT values. Improved osteogenic PKG2 gene expression in aged (12 month old) OB-specific transgenic mice only. Transgene was only detected in the transgenic mice and not in wild-type.

Wild type	Transgenic
No CT	30.485
No CT	30.285
No CT	30.05
No CT	29.41

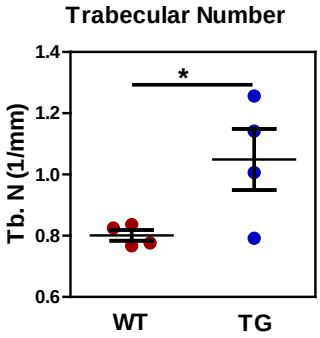
A.



B.



C.



D.

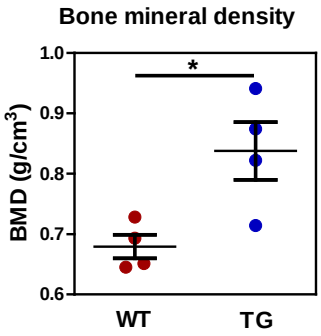
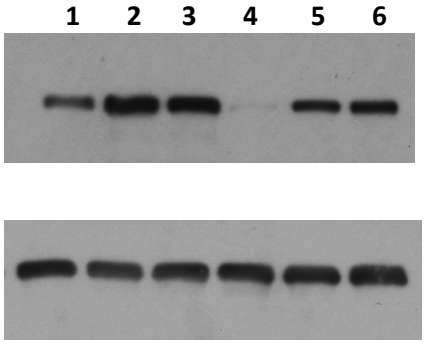


Figure 6. MicroCT- Trabecular bone parameters 12 months wild type versus PKG2 transgenic mice. *A*, Transgenic mice showed improved bone volume to trabecular volume ratio compared to WT aged mice. *B,C* Trabecular thickness and trabecular number is increased in transgenic mice over expressing PKG2 compared to wild type mice. *D*, Bone mineral density was rescued in transgenic mice compared to it deteriorating in the wild type mice.

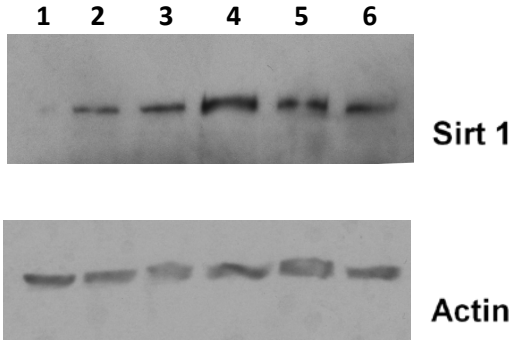
Figure 7: Western blot and RT-qPCR analysis of murine primary osteoblasts. *A,* Western blot analysis comparing pAkt expression in wildtype young and aged mice, treated with the NO/cGMP/PKG 2 signaling pathway activators cinaciguat and NO-cobinamide. *B,* Sirt1 protein expression detected via western blot following a cGMP time course treatment. *C,* RT-qPCR used to determine effects of cGMP treatment on Nrf2 transcription. *D,* RT-qPCR data analyzing differences in Nrf2 transcription in young and aged wild type mice compared to transgenic (osteoblast specific PKG 2 over expressing) mice. *E, ALP staining confirms cells are osteoblasts.*

A.



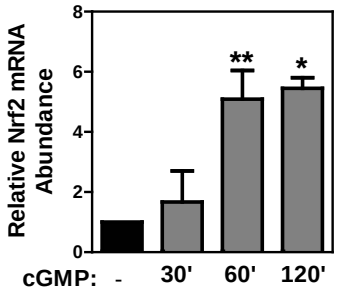
1. 3M WT-1081 Control
2. 3M WT-1081 Cin 100 nM
3. 3M WT-1081 NOCbi 10 μM
4. 12M WT-1 Control
5. 12M WT-1 Cin 100 mM
6. 12M WT-1 NOCbi 10 μM

B.

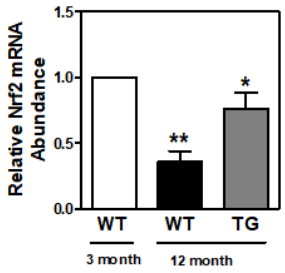


1. Control
2. cGMP 100 μM 3h
3. cGMP 6h
4. cGMP 16h
5. cGMP 24 h
6. Control 2

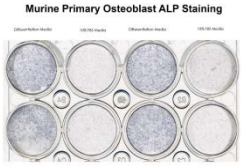
C.



D.



E.



DISCUSSION

Bone is a dynamic organ that undergoes a continuous self-regeneration process called remodeling. To maintain skeletal homeostasis and strength, osteoclasts initiate the remodeling cycle by resorbing mineralized matrix, while osteoblasts form new matrix. An imbalance in bone remodeling —caused by increased bone resorption or decreased bone formation relative to resorption— results in a loss of bone mass, leading to osteoporosis and an increased fracture risk. Aging associated osteoporosis occurs due to a combination of various intrinsic and extrinsic factors resulting in a progressive decline in bone quality. Patients with a reduced bone mass are predisposed to fractures and delayed healing, leading to further complications. The aging process of bone and the pathogenesis of osteoporosis is very closely related and hence there is increased research interest in understanding the mechanisms of age-related bone loss in recent years.

The effects of aging on the development and progression of osteoporosis have shed light on the little known molecular mechanisms of the disease. As individuals age, increased oxidative stress and other cellular stress signals begin to force cells into senescence. Mesenchymal stem cells, osteoblast progenitors, are especially vulnerable to oxidative stress and their senescence results in decreased osteoblast numbers and activity. Osteoclast production continues undisturbed and results in bone loss due to loss of bone homeostasis. Senescence is accompanied by the down regulation of various anabolic pathways and prolierative/antioxidant proteins. Aging is especially associated with a stark increase in oxidative stress, with reactive oxygen and nitrogen species wreaking havoc across the body. In such conditions, the heme group of soluble

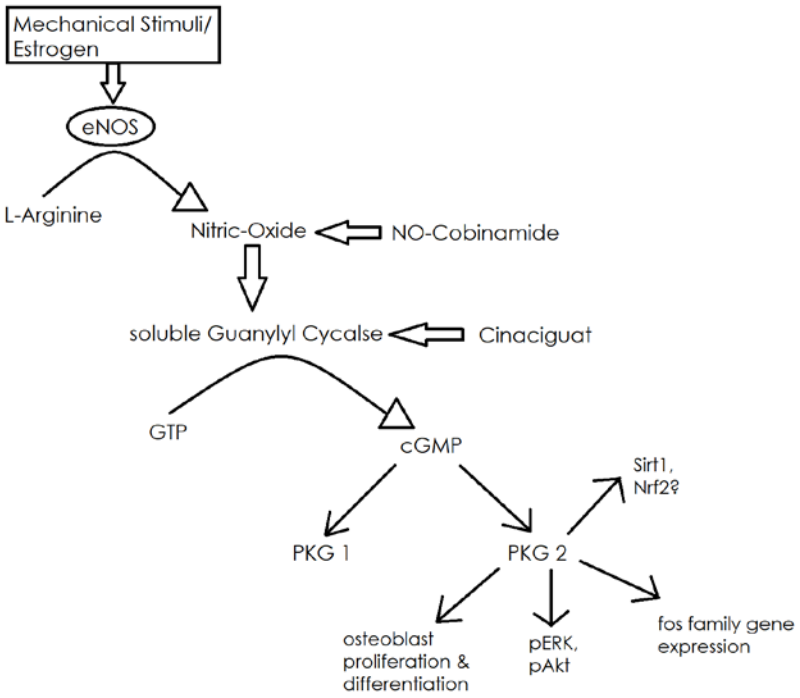
guanylyl cyclase (sGC) becomes oxidized and interferes with the binding and activation by NO (Montfort, 2011). Previous research has shown that NO activation of sGC is impaired in aging mice and so a heme-independent technique for activation of sGC is essential.

Our lab has shown that treatment with cinaciguat enhances PKG II activity via sGC activation under oxidative stress. Additionally, the NO/cGMP/PKG II pathway plays a crucial role in maintaining bone integrity, especially by signaling for osteoblast proliferation and differentiation (Kalyanaraman 2018). My results confirm the previous findings of our laboratory that aged mice have decreased pro-osteoblast and anabolic gene expression and increased osteoclast markers that could be rescued by increased PKG 2 expression in transgenic mice. MicroCT results further confirmed the decreased bone formation in aged mice that could be reversed in transgenic mice. My work went further to show that Sirt1 and Nrf2 activity are decreased with age, and can also be recovered in transgenic mice over expressing PKG II to reverse age induced bone loss. Additionally, treatment with NO-Cbi, Cinaciguat and cGMP was able to increase expression of Runx2, Sirt1 and Nrf2. Taken together, these results suggest that the decrease in BMD with age and increased cellular senescence is caused by down-regulation of Sirt1 protein activity due to diminished signaling through the NO/cGMP/PKG II pathway. The role of PKG II in the proliferation, differentiation and survival of osteoblasts is absolutely essential to maintain bone homeostasis and provides insight into the effects of aging on bone health. With these results in mind, PKG II could be a potential target for drug therapeutics to activate bone forming pathways. However, further investigation is required to uncover its role in Sirt1 activity. As stated

earlier, Sirt1 plays a crucial role in numerous pathways throughout the body, most of which have a proliferative and anti-aging effect. Due to its complex functions, the protein is also under a complex transcriptional and translational regulatory system controlled by several proteins. It will be important to uncover where exactly PKG II plays a role in this cobweb of regulation. Nrf2 is a promising factor due to its role in bone anabolic pathways. Chemical factors that can block or improve the nuclear import of Nrf2 can be used to evaluate whether it is in fact a downstream target of PKG II. It would also be important to perform an RNA-sequencing analysis to get a global perspective of gene expression profiles in young versus aged mice and to identify potential PKG targets that regulate osteogenesis. The extensive anti-aging role played by Sirt1 throughout the body and not just in bone tissue, prompts experiments far greater than the scope of my research. As an important anti-oxidative protein, it would be interesting to modulate Sirt1 action by activating cGMP/PKG II and examining whether there is improved mitochondrial function and decreased oxidative stress in aged bone marrow stromal cells.

Overall, we have shown that multiple steps of the NO/cGMP/PKG signaling pathway is defective in aged osteoblasts. Additionally, levels of the ultra-important anti-senescence protein, Sirt1, along with expression of its transcriptional regulator Nrf2, are dramatically down regulated in aging cells. Cinaciguat and NO-Cbi are potential therapeutic agents for increasing bone formation and treating aging-associated osteoporosis.

Table 2. NO/cGMP/PKG Signaling Pathway. A simplification of the signal transduction pathway focusing on areas relevant to the experiments. Further experiments are required to confirm Nrf2 and Sirt1 as downstream effectors of the pathway.



REFERENCES

Bone health and osteoporosis: A report of the Surgeon General. (2004). Rockville, MD: U.S. Dept. of Health and Human Services, Public Health Service, Office of the Surgeon General.

Abdelmohsen, K., Pullmann, R., Lal, A., Kim, H. H., Galban, S., Yang, X., Gorospe, M. (2007). Phosphorylation of HuR by Chk2 Regulates SIRT1 Expression. *Molecular Cell, 25*(4), 543-557.

Almeida, M., & O'brien, C. A. (2013). Basic Biology of Skeletal Aging: Role of Stress Response Pathways. *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences, 68*(10), 1197-1208. doi:10.1093/gerona/glt079

Armour, K. E., Armour, K. J., Gallagher, M. E., Gödecke, A., Helfrich, M. H., Reid, D. M., & Ralston, S. H. (2001). Defective Bone Formation and Anabolic Response to Exogenous Estrogen in Mice with Targeted Disruption of Endothelial Nitric Oxide Synthase1. *Endocrinology, 142*(2), 760-766. doi:10.1210/endo.142.2.7977

Bai, X., Lu, D., Bai, J., Zheng, H., Ke, Z., Li, X., & Luo, S. (2004, January 30). Oxidative stress inhibits osteoblastic differentiation of bone cells by ERK and NF-κB. *Biochemical and Biophysical Research Communications, 314*(1), 197-207. doi:10.1016/j.bbrc.2003.12.073

Brennan, T. A., Egan, K. P., Lindborg, C. M., Chen, Q., Sweetwyne, M. T., Hankenson, K. D., Pignolo, R. J. (2014). Mouse models of telomere dysfunction phenocopy skeletal changes found in human age-related osteoporosis. *Disease Models & Mechanisms, 7*(5), 583-592. doi:10.1242/dmm.014928

Corpas, R., Revilla, S., Ursulet, S., Castro-Freire, M., Kaliman, P., Petegnief, V., Sanfeliu, C. (2016). SIRT1 Overexpression in Mouse Hippocampus Induces Cognitive Enhancement Through Proteostatic and Neurotrophic Mechanisms. *Molecular Neurobiology, 54*(7), 5604-5619.

Daitoku, H., Hatta, M., Matsuzaki, H., Aratani, S., Ohshima, T., Miyagishi, M., Fukamizu, A. (2004). Silent information regulator 2 potentiates Foxo1-mediated transcription through its deacetylase activity. *Proceedings of the National Academy of Sciences, 101*(27), 10042-10047.

Edwards, J. R., Perrien, D. S., Fleming, N., Nyman, J. S., Ono, K., Connelly, L., Elefteriou, F. (2013). Silent information regulator (Sir)T1 inhibits NF-κB signaling to maintain normal skeletal remodeling. *Journal of Bone and Mineral Research, 28*(4), 960-969.

Fei, Y., Shimizu, E., Mcburney, M. W., & Partridge, N. C. (2015). Sirtuin 1 Is a Negative Regulator of Parathyroid Hormone Stimulation of Matrix Metalloproteinase 13 Expression in Osteoblastic Cells. *Journal of Biological Chemistry, 290*(13), 8373-8382.

Fox, S., & Chow, J. (1998). Nitric Oxide Synthase Expression in Bone Cells. *Bone, 23*(1), 1-6. doi:10.1016/s8756-3282(98)00070-2

Fritz, B. G., Hu, X., Brailey, J. L., Berry, R. E., Walker, F. A., & Montfort, W. R. (2011). Oxidation and Loss of Heme in Soluble Guanylyl Cyclase fromManduca sexta. *Biochemistry, 50*(26), 5813-5815.

Geiss-Friedlander, R., & Melchior, F. (2007). Concepts in sumoylation: A decade on. *Nature Reviews Molecular Cell Biology, 8*(12), 947-956.

Hof, R. V., & Ralston, S. (1995). Cytokine-induced nitric oxide production inhibits bone resorption in mouse bone marrow cultures by inducing apoptosis in osteoclast progenitors. *Bone, 17*(6), 587. doi:10.1016/8756-3282(96)87904-x

Hukkanen, M., Platts, L., Lawes, T., Girgis, S., Konttinen, Y., Goodship, A., Polak, J. M. (2003). Effect of nitric oxide donor nitroglycerin on bone mineral density in a rat model of estrogen deficiency-induced osteopenia. *Bone, 32*(2), 142-149. doi:10.1016/s8756-3282(02)00955-9

Iida, H., & Fukuda, S. (2002). Age-Related Changes in Bone Mineral Density, Cross-Sectional Area and Strength at Different Skeletal Sites in Male Rats. *Journal of Veterinary Medical Science, 64*(1), 29-34. doi:10.1292/jvms.64.29

Iyer, S., Han, L., Bartell, S. M., Kim, H., Gubrij, I., Cabo, R. D., Almeida, M. (2014). Sirtuin1 (Sirt1) Promotes Cortical Bone Formation by Preventing β-Catenin Sequestration by FoxO Transcription Factors in Osteoblast Progenitors. *Journal of Biological Chemistry, 289*(35), 24069-24078.

Joshua, J., Kalyanaraman, H., Marathe, N., & Pilz, R. B. (2014). Nitric Oxide as a Mediator of Estrogen Effects in Osteocytes. *Vitamins & Hormones Nitric Oxide,* 247-263.

Kalyanaraman, H., Ramdani, G., Joshua, J., Schall, N., Boss, G. R., Cory, E., Pilz, R. B. (2016). A Novel, Direct NO Donor Regulates Osteoblast and Osteoclast Functions and Increases Bone Mass in Ovariectomized Mice. *Journal of Bone and Mineral Research, 32*(1), 46-59. doi:10.1002/jbmr.2909

Kalyanaraman, H., Schwaerzer, G., Ramdani, G., Castillo, F., Scott, B. T., Dillmann, W., Pilz, R. B. (2018). Protein Kinase G Activation Reverses Oxidative Stress and Restores Osteoblast Function and Bone Formation in Male Mice With Type 1 Diabetes. *Diabetes, 67*(4), 607-623. doi:10.2337/db17-0965

Kim, J., Singhal, V., Biswal, S., Thimmulappa, R. K., & Digirolamo, D. J. (2014). Nrf2 is required for normal postnatal bone acquisition in mice. *Bone Research, 2*(1).

Kwon, H., & Ott, M. (2008). The ups and downs of SIRT1. *Trends in Biochemical Sciences, 33*(11), 517-525.

Luo, J., Nikolaev, A. Y., Imai, S., Chen, D., Su, F., Shiloh, A., Gu, W. (2001). Negative Control of p53 by Sir2α Promotes Cell Survival under Stress. *Cell, 107*(2), 137-148.

Ma, Q. (2013). Role of Nrf2 in Oxidative Stress and Toxicity. *Annual Review of Pharmacology and Toxicology, 53*(1), 401-426.

Manolagas, S. C. (2000). Birth and Death of Bone Cells: Basic Regulatory Mechanisms and Implications for the Pathogenesis and Treatment of Osteoporosis. *Endocrine Reviews, 21*(2), 115-137. doi:10.1210/er.21.2.115

Marathe, N., Rangaswami, H., Zhuang, S., Boss, G. R., & Pilz, R. B. (2011). Pro-survival Effects of 17β-Estradiol on Osteocytes Are Mediated by Nitric Oxide/cGMP via Differential Actions of cGMP-dependent Protein Kinases I and II. *Journal of Biological Chemistry, 287*(2), 978-988.

Mattagajasingh, I., Kim, C., Naqvi, A., Yamamori, T., Hoffman, T. A., Jung, S., Irani, K. (2007). SIRT1 promotes endothelium-dependent vascular relaxation by activating endothelial nitric oxide synthase. *Proceedings of the National Academy of Sciences, 104*(37), 14855-14860.

Michishita, E., Park, J. Y., Burneskis, J. M., Barrett, J. C., & Horikawa, I. (2005). Evolutionarily Conserved and Nonconserved Cellular Localizations and Functions of Human SIRT Proteins. *Molecular Biology of the Cell, 16*(10), 4623-4635.

Montfort, W. R., Wales, J. A., & Weichsel, A. (2017). Structure and Activation of Soluble Guanylyl Cyclase, the Nitric Oxide Sensor. *Antioxidants & Redox Signaling, 26*(3), 107-121. doi:10.1089/ars.2016.6693

Movérare-Skrtic, S., Henning, P., Liu, X., Nagano, K., Saito, H., Börjesson, A. E., Ohlsson, C. (2014). Osteoblast-derived WNT16 represses osteoclastogenesis and prevents cortical bone fragility fractures. *Nature Medicine, 20*(11), 1279-1288.

Nisoli, E. (2005). Calorie Restriction Promotes Mitochondrial Biogenesis by Inducing the Expression of eNOS. *Science, 310*(5746), 314-317.

Ota, H., Eto, M., Kano, M. R., Kahyo, T., Setou, M., Ogawa, S., Ouchi, Y. (2010). Induction of Endothelial Nitric Oxide Synthase, SIRT1, and Catalase by Statins Inhibits Endothelial Senescence Through the Akt Pathway. *Arteriosclerosis, Thrombosis, and Vascular Biology, 30*(11), 2205-2211.

Parker, J. D. (2004). Nitrate tolerance, oxidative stress, and mitochondrial function: Another worrisome chapter on the effects of organic nitrates. *Journal of Clinical Investigation, 113*(3), 352-354.

Rangaswami, H., Schwappacher, R., Marathe, N., Zhuang, S., Casteel, D. E., Haas, B., Pilz, R. B. (2010). Cyclic GMP and Protein Kinase G Control a Src-Containing Mechanosome in Osteoblasts. *Science Signaling, 3*(153).

Rangaswami, H., Schwappacher, R., Tran, T., Chan, G. C., Zhuang, S., Boss, G. R., & Pilz, R. B. (2012). Protein Kinase G and Focal Adhesion Kinase Converge on Src/Akt/β-Catenin Signaling Module in Osteoblast Mechanotransduction. *Journal of Biological Chemistry, 287*(25), 21509-21519. doi:10.1074/jbc.m112.347245

Reid, I. R. (2015). Short-term and long-term effects of osteoporosis therapies. *Nature Reviews Endocrinology, 11*(7), 418-428.

Riggs, B. L., Khosla, S., & Melton, L. J. (2002). Sex Steroids and the Construction and Conservation of the Adult Skeleton. *Endocrine Reviews, 23*(3), 279-302. doi:10.1210/edrv.23.3.0465

Rogina, B., & Helfand, S. L. (2004). Sir2 mediates longevity in the fly through a pathway related to calorie restriction. *Proceedings of the National Academy of Sciences, 101*(45), 15998-16003.

Shan, J., He, H., Li, M., Zhu, J., Cheng, Y., Hu, N., Yang, Z. (2015). APE1 promotes antioxidant capacity by regulating Nrf-2 function through a redox-dependent mechanism. *Free Radical Biology and Medicine, 78*, 11-22.

Syed, F. A., Mödder, U. I., Roforth, M., Hensen, I., Fraser, D. G., Peterson, J. M., Khosla, S. (2010). Effects of chronic estrogen treatment on modulating age-related bone loss in female mice. *Journal of Bone and Mineral Research, 25*(11), 2438-2446. doi:10.1002/jbmr.129

Thomas, G. R., Difabio, J. M., Gori, T., & Parker, J. D. (2007). Once Daily Therapy With Isosorbide-5-Mononitrate Causes Endothelial Dysfunction in Humans. *Journal of the American College of Cardiology, 49*(12), 1289-1295.

Tian, W., Vega, M. R., Schmidlin, C. J., Ooi, A., & Zhang, D. D. (2017). Kelch-like ECH-associated protein 1 (KEAP1) differentially regulates nuclear factor erythroid-2–related factors 1 and 2 (NRF1 and NRF2). *Journal of Biological Chemistry, 293*(6), 2029-2040.

Tissenbaum, H. A., & Guarente, L. (2001). Increased dosage of a sir-2 gene extends lifespan in Caenorhabditis elegans. *Nature, 410*(6825), 227-230.

Ucer, S., Iyer, S., Kim, H., Han, L., Rutlen, C., Allison, K., Manolagas, S. C. (2016). The Effects of Aging and Sex Steroid Deficiency on the Murine Skeleton Are Independent and Mechanistically Distinct. *Journal of Bone and Mineral Research, 32*(3), 560-574. doi:10.1002/jbmr.3014

Yan Dai And Douglas V. Faller. (2008). Transcription Regulation by Class III Histone Deacetylases (HDACs)—Sirtuins. *Translational Oncogenomics, 1*, 53-65. doi:10.4137/tog.s483

Yang, Y., Fu, W., Chen, J., Olashaw, N., Zhang, X., Nicosia, S. V., Bai, W. (2007). SIRT1 sumoylation regulates its deacetylase activity and cellular response to genotoxic stress. *Nature Cell Biology, 9*(11), 1253-1262.

Yeung, F., Hoberg, J. E., Ramsey, C. S., Keller, M. D., Jones, D. R., Frye, R. A., & Mayo, M. W. (2004). Modulation of NF-κB-dependent transcription and cell survival by the SIRT1 deacetylase. *The EMBO Journal, 23*(12), 2369-2380.

Yoon, D. S., Choi, Y., & Lee, J. W. (2016). Cellular localization of NRF2 determines the self-renewal and osteogenic differentiation potential of human MSCs via the P53–SIRT1 axis. *Cell Death & Disease, 7*(2).

Zainabadi, K., Liu, C. J., & Guarente, L. (2017). SIRT1 is a positive regulator of the master osteoblast transcription factor, RUNX2. *Plos One, 12*(5).