Rapamycin impairs bone accrual in young adult mice independent of Nrf2

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\section{Introduction}

Age-related skeletal pathologies represent a major public health concern affecting approximately 60\% of adults >50 years of age \cite{1}. Bone fractures in aged adults increase the risk of mortality and create a significant economic burden on the health care system, as they require long recovery periods \cite{2,3}. The skeleton is constantly remodeled throughout the lifespan and bone homeostasis requires an equal balance of bone resorption by osteoclasts and bone formation by osteoblasts \cite{4}. During normal aging bone resorption slightly exceeds bone formation, ultimately leading to a progressive decrease in bone mass and bone quality, and increased skeletal fragility \cite{5}.

The mammalian target of rapamycin (mTOR) is a serine/threonine kinase that plays a central role in cell growth, nutrient sensing, and energy metabolism \cite{6,7}. The mTOR signaling pathway has emerged as an important mediator of the aging process and the etiology of aging-related pathologies, based on the finding that the mTOR inhibitor rapamycin increases health span and lifespan in a variety of model organisms, including several mouse strains \cite{8-10}. In vitro and in vivo genetic studies demonstrate a mechanistic role for mTOR in skeletal development, skeletal stem cell fate determination, bone cell activity, and fracture healing \cite{11-18}. Limited studies, with mixed results, have examined the in vivo impact of rapamycin on bone cell metabolism \cite{19-25}.
The number of preclinical studies investigating rapamycin as a therapy to attenuate aging and aging-related pathologies is rapidly growing, and several clinical trials are underway to determine the safety and efficacy of rapamycin and other mTOR inhibitors as anti-aging interventions in humans [26–28]. It is likely the effects of rapamycin treatment are not equivalent throughout different life phases, and a critical component of these studies is determining the impact of rapamycin treatment from skeletal development through senescence. This is particularly important if rapamycin were used as countermeasure to treat normal or accelerated aging. As such, understanding the impact of rapamycin on the skeleton of both healthy adult mice and experimental mouse models of aging may be useful for translating preclinical data to the clinic.

In this study we sought to determine the impact of rapamycin treatment on bone mass, microarchitecture, turnover, and inflammatory status in adult wild type mice and mice with a global deletion of transcription factor nuclear factor (erythroid-derived 2)-like 2 (Nrf2 or NFE2L2). Nrf2 is a central regulator of redox homeostasis through the regulation of the antioxidant gene response [29]. Mice lacking Nrf2 are an excellent model of aging-related disease vulnerability, as they have a reduced lifespan, dramatically increased levels of cellular oxidative stress, senescence, and inflammation, all of which correspond to higher incidence of aging-related diseases including cancer, cardiovascular pathology, inflammatory conditions, neurodegeneration, and skeletal impairment [29–33].

2. Materials and methods

2.1. Experimental design

8-week-old female wild-type (ICR, n = 15) and Nrf2−/− mice (ICR background, n = 18) were obtained from an in-house breeding colony at Oregon State University. The Nrf2−/− breeding colony founders were obtained from Dr. Masayuki at the Yamamoto Kohoku University of Japan. Mice were housed under controlled conditions with approval of the Oregon State University Institutional Animal Care and Use Committee. Mice were individually housed in a room on standard light cycle (12 h light; 12 h dark), and food and water were provided ad libitum. Body weight and food consumption were monitored weekly for the duration of the study.

Mice were genotyped at approximately 18 days of age and separated at weaning. At age 16–20 weeks old, WT and Nrf2−/− mice were randomized into one of two treatment conditions, rapamycin or vehicle. Mice treated with rapamycin received intraperitoneal injection of rapamycin (dissolved in ethanol then diluted with vehicle containing 5% PEG4000) at a dosage of 4 mg/kg [34]. The dosing was given every other day for a duration of 12 weeks. Vehicle-treated mice received intraperitoneal injections of equimolar volume of saline (with 5% Tween 80 and 5% PEG4000).

2.2. Dual energy X-ray absorptiometry

Total femur bone mineral content (BMC, g), area (cm²), and bone mineral density (BMD, g/cm²) were measured ex vivo using dual energy absorptiometry (DXA; Piximus 2, Lunar Corp., Madison, WI).

2.3. Microcomputed tomography

Microcomputed tomography (µCT) was used for nondestructive three-dimensional evaluation of bone volume and architecture in femur and 5th lumbar vertebra as previously described [35].

2.4. Histomorphometry

Methods for measuring static and dynamic bone histomorphometry in distal femur metaphysis have been described in detail [35–37]. Adipocyte density and size were measured in the distal femur metaphysis using Adobe Photoshop (Adobe Systems, San Jose, CA). Digital images of the distal femur metaphysis were captured under ultraviolet conditions and adipocytes were identified as large circular or oval-shaped cells bordered by a prominent cell membrane. Adipocytes density was measured using the counting tool and adjusted for tissue area. Adipocyte size was measured using the lasso tool.

2.5. Serum biochemistry

Serum osteocalcin was measured using Mouse Gla-Osteocalcin High Sensitive ELISA Kit (Clontech, Mountain View, CA) and serum CTX-1 was measured using Mouse CTX-1 ELISA kit (LifeSpan Biosciences, Seattle, WA) according to the respective manufacturer’s protocol.

2.6. Gene expression

Tibiae were pulverized in liquid nitrogen and homogenized in Trizol (Life Technologies, Grand Island, NY). Total RNA was isolated using a Qiagen RNaseasy Mini Kit (Qiagen, Germantown, MD) according to the manufacturer’s protocol. RNA was reverse transcribed into cDNA using High Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific, Waltham, MA). qRT-PCR was conducted on an Applied Biosystems 7500 HT platform using Fast SYBR Green Master Mix. Gene expression for MCP-1, ICAM, VCAM, IL-6, TNA-alpha, and IL-1beta was determined using the following primer sequences.

| Gene               | Forward primer            | Reverse primer            |
|--------------------|---------------------------|--------------------------|
| Cd2 (MCP-1)        | TCCCAATGAGTGGCTGGA        | TCTGGAACCAATCCCTCCTCTG   |
| Icam1 (ICAM)       | TCCACGGATGAGCAGCCTC       | GTCTGGTGAGACCCCTCTTG     |
| Vcam1 (VCAM)       | CCGCCTCTGGAGAATATTGG      | TAGACCCTGGTGAAAGCGCTG    |
| Il6 (IL-6)         | GAGGATACCTCCCACAGGACC    | AGTCGATCATGTTGCTCTCTACA  |
| Tnf (TNF-alpha)    | CTGAGCCCAAGCTGCTAGCA      | GTGGTTGGAAGGACGCTA       |
| Il1b (IL-1 beta)   | AAGTGAAGGGCCGCTTCCCAA     | TGAAGAAGAAAAGAGGCTTCATG  |
| Rn18s (18s rRNA)   | CCGCAGCTGGAAATAGGGAAT    | GAAGAATGGAGGCTTCTCTCT   |
| Gapdh              | TCCACTCCTTCCACATTCTCGA   | AGTTGGGATAGGGCCCTCTCTG  |

Gene expression was normalized to 18S and GAPDH, and relative quantification was determined (ΔΔCt).

The mice were injected with the fluorochrome calcein (15 mg/kg, Sigma, USA) 4 days and 1 day prior to sacrifice to label bone surfaces undergoing mineralization. For tissue collection, mice were deeply anesthetized with 2% isoflurane and bled by decapitation. Right femora and 5th lumbar vertebrae were isolated, fixed for 24 h in 10% formalin, and stored in 70% ethanol for microcomputed tomography and histomorphometric analyses. Left and right tibiae were removed, snap frozen in liquid nitrogen, and stored at −80 °C for RNA analysis of genes involved in inflammatory processes.
2.7. Statistics

A two-factor linear model with an interaction between treatment (rapamycin or vehicle control) and genotype (WT and Nrf2*−/−) was used to compare mean values for bone densitometry, architecture, and histomorphometry endpoints. Residual analysis, Levene's test for homogeneity of variance, and Anderson-Darling tests of normality were used to assess goodness of model fit. The Benjamini and Hochberg method for maintaining the false discovery rate at 5% was used to adjust for multiple comparisons [38]. Differences were considered significant at p ≤ 0.05. All data are presented as mean ± SE. Data analysis was performed using R version 3.4.3.

3. Results

There were no differences in ages between experimental groups, and neither rapamycin treatment (4 mg/kg administered intraperitoneally every other day for 12 weeks) nor genotype impacted terminal body mass. We utilized DXA to assess bone mass and density in the whole femur. Mice treated with rapamycin exhibited significantly lower femoral bone mineral content and density compared to vehicle-treated mice (Table 1). In agreement with a prior study, global deletion of the Nrf2 gene had no impact on bone mineral content or density [39]. Additionally, there was no interaction in the response to rapamycin between wild-type and Nrf2*−/− mice. Bone area did not differ with genotype or rapamycin treatment. However, rapamycin treatment resulted in lower total femur BMC, BMD and bone volume, irrespective of genotype.

Bone microarchitecture is an indicator of bone health and predicts fracture risk. We used uCT to assess bone length, total bone volume, and cortical and cancellous bone microarchitecture in the femur and cancellous bone microarchitecture in 5th lumbar vertebra (Table 1). The initiation of rapamycin treatment was timed to the latter stage of linear growth and bone accrual [40]. Assessment of the femoral growth plate confirmed the mice were no longer growing at the study completion. Rapamycin treatment did not affect bone length and there was no difference in bone length between wild type and Nrf2*−/− mice.

Cortical, or compact bone, comprises ~80% of total body bone mass in humans and primarily provides mechanical strength against compressive forces. To determine the impact of rapamycin on cortical bone, we assessed bone microarchitecture in the mid femur diaphysis. Mice treated with rapamycin exhibited lower cortical bone volume and cortical thickness compared to vehicle-treated mice. There was no effect of rapamycin on marrow volume and there were no independent effects of Nrf2 KO on cortical parameters. The polar moment of inertia, an indirect index of bone strength in torsion, tended to be lower (P = 0.071) in rapamycin-treated mice. There were no genotype × treatment interactions for the measured endpoints.

Cancellous, or trabecular bone, accounts for ~20% of total bone mass in humans and much less in mice and functions, in part, to distribute mechanical loads to the stronger cortical bone, and because of its large surface/volume ratio plays a critical role in mineral homeostasis. Cancellous bone has a much higher turnover rate compared to cortical bone, and as such, cancellous bone is preferentially lost during aging and metabolic skeletal disease. We assessed cancellous bone in the distal femur metaphysis. Rapamycin treatment resulted in lower trabecular connectivity density and higher trabecular spacing. There was no independent effect of genotype and no genotype × treatment

Table 1

| Variable | Wild-type mice | Nrf2*−/− mice | Genotype | Rapamycin | Genotype × rapamycin |
|----------|----------------|---------------|----------|-----------|---------------------|
|          | Vehicle (n = 7) | Rapamycin (n = 8) | Vehicle (n = 8) | Rapamycin (n = 10) | P value | P value | P value |
| Age at termination (weeks) | 27.9 ± 0.7 | 31.4 ± 1.8 | 29.1 ± 1.1 | 29.2 ± 1.6 | NS | One-way ANOVA | NS | NS | NS |
| Body mass at termination (g) | 31.1 ± 1.0 | 31.5 ± 1.0 | 32.4 ± 1.1 | 32.0 ± 0.6 | NS | NS | NS |
| Dual energy X-ray absorptiometry |          |               |           |             |       |       |       |
| Total femur |                 |               |           |             |       |       |       |
| Bone area (cm²) | 0.51 ± 0.01 | 0.51 ± 0.01 | 0.51 ± 0.01 | 0.49 ± 0.01 | NS | NS | NS |
| BMC (g) | 0.034 ± 0.001 | 0.035 ± 0.001 | 0.032 ± 0.001 | 0.031 ± 0.00 | NS | 0.014 | NS |
| BMD (g/cm²) | 0.67 ± 0.001 | 0.068 ± 0.002 | 0.062 ± 0.002 | 0.063 ± 0.001 | NS | 0.003 | NS |
| Micro-computed tomography |          |               |           |             |       |       |       |
| Total femur |                 |               |           |             |       |       |       |
| Bone volume (mm³) | 28.5 ± 0.7 | 28.8 ± 0.7 | 26.8 ± 1.0 | 25.8 ± 0.4 | NS | 0.014 | NS |
| Length (mm) | 16.5 ± 0.2 | 16.4 ± 0.1 | 16.4 ± 0.2 | 16.1 ± 0.1 | NS | NS | NS |
| Femur diaphysis (cortical bone) |                 |               |           |             |       |       |       |
| Cross-sectional volume (mm²) | 0.46 ± 0.02 | 0.047 ± 0.01 | 0.44 ± 0.02 | 0.44 ± 0.01 | NS | NS | NS |
| Cortical volume (mm³) | 0.29 ± 0.01 | 0.29 ± 0.01 | 0.27 ± 0.01 | 0.27 ± 0.01 | NS | 0.034 | NS |
| Marrow volume (mm³) | 0.17 ± 0.01 | 0.18 ± 0.01 | 0.17 ± 0.01 | 0.17 ± 0.00 | NS | NS | NS |
| Cortical thickness (μm) | 293 ± 7 | 293 ± 7 | 277 ± 6 | 276 ± 4 | NS | 0.029 | NS |
| I² polar (mm²) | 0.50 ± 0.03 | 0.52 ± 0.03 | 0.46 ± 0.03 | 0.45 ± 0.02 | NS | NS (0.071) | NS |
| Femur metaphysis (cancellous bone) |                 |               |           |             |       |       |       |
| Bone volume/tissue volume (%) | 11.0 ± 1.5 | 9.6 ± 1.1 | 8.7 ± 1.5 | 6.5 ± 0.9 | NS | NS | NS |
| Trabecular connectivity density (1/mm²) | 34.4 ± 4.2 | 25.0 ± 5.1 | 21.8 ± 4.9 | 15.8 ± 3.6 | NS | 0.050 | NS |
| Trabecular number (1/mm) | 3.8 ± 0.1 | 3.8 ± 1.1 | 3.7 ± 0.1 | 3.5 ± 0.1 | NS | NS | NS |
| Trabecular thickness (μm) | 62 ± 4 | 63 ± 2 | 62 ± 3 | 60 ± 1 | NS | NS | NS |
| Trabecular spacing (μm) | 274 ± 6 | 279 ± 9 | 290 ± 5 | 299 ± 7 | NS | 0.045 | NS |
| 5th lumbar vertebra |                 |               |           |             |       |       |       |
| Total vertebra |                 |               |           |             |       |       |       |
| Bone volume (mm³) | 8.4 ± 0.4 | 8.2 ± 0.4 | 7.8 ± 0.4 | 7.3 ± 0.2 | NS | 0.047 | NS |
| Vertebral body (cancellous bone) |                 |               |           |             |       |       |       |
| Bone volume/tissue volume (%) | 24.1 ± 1.8 | 24.1 ± 1.8 | 22.4 ± 1.6 | 19.0 ± 0.7 | NS | 0.034 | NS |
| Trabecular connectivity density (1/mm²) | 98.3 ± 5.1 | 98.0 ± 5.8 | 94.0 ± 7.1 | 91.2 ± 7.6 | NS | NS | NS |
| Trabecular number (1/mm) | 3.6 ± 0.2 | 3.6 ± 0.2 | 3.4 ± 0.2 | 3.4 ± 0.1 | NS | NS | NS |
| Trabecular thickness (μm) | 62 ± 2 | 64 ± 2 | 59 ± 2 | 57 ± 1 | NS | 0.027 | NS |
| Trabecular spacing (μm) | 292 ± 7 | 295 ± 15 | 304 ± 18 | 301 ± 11 | NS | NS | NS |
interactions were detected for any of the endpoints measured.

To determine whether the observed effects of rapamycin on femur were generalizable to the axial skeleton, we assessed the impact of rapamycin on cancellous bone in the lumbar vertebra. Skeletal sites with a high ratio of cancellous to cortical bone, such as the vertebra, are common sites of osteoporotic and aging-related fractures in humans. Mice treated with rapamycin had lower total vertebral bone volume, and lower cancellous bone volume fraction and trabecular thickness in the vertebral body compared to vehicle-treated mice. There was no effect of rapamycin on trabecular connectivity density, number, or spacing, and there was no independent effect of genotype on any of the cancellous bone parameters. Additionally, no genotype × treatment interaction was detected.

Bone histomorphometry is considered the gold standard methodology for assessing bone turnover at the local level [36]. We analyzed static and dynamic bone histomorphometry in distal femur metaphysis histological sections to evaluate the impact of rapamycin on cellular processes regulating bone turnover (Fig. 1). Significant differences in mineralizing perimeter and osteoblast perimeter, an index of osteoblast number, were not detected with genotype or treatment (Fig. 1A and D).

Mineral apposition rate, an index of osteoblast activity, was lower in rapamycin-treated mice (Fig. 1B). Bone formation rate normalized to bone perimeter was also lower in rapamycin-treated mice compared to vehicle-treated mice (Fig. 1C). Rapamycin treatment had no impact on serum osteocalcin, a marker of global bone formation, suggesting that reduced bone formation rate in the femur metaphysis was location specific (Fig. 1F). Osteoclast perimeter, an index of osteoclast number, was not different between treatment groups (Fig. 1E); however, serum CTX-1, a marker of global bone resorption, was higher in rapamycin-treated mice compared to vehicle-treated mice suggesting that osteoclast activity, rather than osteoclast number, may be increased by rapamycin (Fig. 1G). Alternatively, the effect of rapamycin treatment on osteoclast number could be site specific. There were no independent effects of genotype on histomorphometry parameters and no genotype × treatment interactions were detected. The impact of rapamycin on bone formation in the distal femur metaphysis can be observed in Fig. 2.

Bone marrow mesenchymal stem cells can differentiate into osteoblasts, adipocytes, or chondrocytes [41]. Differentiation fate is driven by local and systemic stimuli, and the balance of osteoblast and adipocyte differentiation is believed to be an important factor in the maintenance

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**Fig. 1.** Effects of rapamycin and Nrf2 KO on femur histomorphometry and serum biomarkers of bone turnover in adult female mice. *p ≤ 0.05 for treatment main effect; *p ≤ 0.05 for genotype main effect.
of bone homeostasis \cite{41}. We used static histomorphometry to assess the impact of rapamycin on bone marrow adiposity. Rapamycin treatment did not impact bone marrow adipocyte density or adipocyte size (Fig. 1H), but there was a genotype effect, where Nrf2\textsuperscript{−/−} mice exhibited larger adipocyte size compared to WT mice (Fig. 1I). No genotype × treatment interaction was detected.

Rapamycin has been shown to reduce inflammation in numerous cells and tissues, and this attenuation is dependent, in part, on Nrf2. To determine whether rapamycin treatment influenced the inflammatory status of bone, we assessed gene expression of prototypical proinflammatory chemokines MCP1, ICAM, VCAM in tibia from Nrf2\textsuperscript{−/−} mice treated with rapamycin for 12 weeks, which is comprised of both bone marrow and bone. Additionally, we also found no rapamycin-induced reduction in inflammatory markers in tibia from Nrf2\textsuperscript{−/−} mice, which had significantly elevated inflammation. The exact reason bone and/or bone marrow is refractory to rapamycin-induced alteration in inflammatory status is unclear.

A key aspect to evaluating the clinical utility of rapamycin as an anti-aging therapy is determining the differential effects of rapamycin throughout the lifespan, and in particular, understanding how the age or ‘life phase’ of rapamycin treatment initiation influences health and longevity. Studies investigating the impact of rapamycin on bone metabolism in rodents have produced varying results, and we speculate the discrepancies may be due, in part, to the age at which rapamycin treatment was initiated. In young rodents, rapamycin treatment retards bone growth through disruption in growth plate dynamics and endochondral ossification \cite{19,20,23,24}. In the present study, we chose to initiate rapamycin treatment at 16–20 weeks of age – an interval shortly before termination of skeletal growth – to establish the effects of rapamycin on development of peak bone mass, a major determinant of bone health during aging. Based on our findings, we conclude that rapamycin treatment has adverse effects at this critical stage of skeletal maturation. In contrast to our findings, the few studies examining rapamycin or rapamycin analogs in older rodents have reported moderate protective effects of rapamycin against bone loss, suggesting the impact of rapamycin on skeletal health may be age-or context-specific \cite{21,22,25}.

**Fig. 2.** Representative histological images of the distal femur metaphysis illustrating differences in fluorochrome labeling with rapamycin treatment. The distance between the distinct fluorochrome labels is used to determine mineral apposition rate.

**Fig. 3.** Effects of rapamycin and Nrf2 KO on tibia inflammatory gene expression. Nrf2\textsuperscript{−/−} mice had significantly higher expression of the inflammatory chemokines MCP1, ICAM, VCAM compared to WT mice. There was no significant independent effect of rapamycin or interaction between rapamycin and genotype. \( p \leq 0.05 \) for genotype main effect.

4. Discussion

Adult WT and Nrf2\textsuperscript{−/−} mice treated with rapamycin for 12 weeks, compared to vehicle-treated mice, exhibited lower total femur bone mineral density, cortical bone volume and thickness, and cancellous bone volume fraction in lumbar vertebra, with no change in body weight. At the cellular level, the deleterious effects of rapamycin treatment were associated with net decrease in bone formation rate in distal femur metaphysis. Nrf2 status did not influence the impact of rapamycin on bone metabolism in adult mice.

The actions and mechanisms through which rapamycin influences bone metabolism in mice are not well established. Maintenance of bone homeostasis depends on the balance of bone resorption by osteoclasts and bone formation by osteoblasts. Osteoblasts are derived from bone marrow mesenchymal stem cells, which can also differentiate into adipocytes \cite{42}. In the present study, we found no rapamycin-induced differences in osteoblast perimeter or mineralizing perimeter per bone perimeter; there were also no differences in adipocyte number or density between rapamycin and vehicle-treated mice. Thus, redirection of MSC differentiation from osteoblasts to adipocytes is unlikely to contribute to decreased bone formation in rapamycin-treated mice. There is in vitro evidence that rapamycin impairs the differentiation of pre-osteoblasts into mature osteoblasts \cite{17} and mice treated with rapamycin exhibited lower MAR, an index of osteoblast activity, compared to vehicle-treated mice. Coupled to higher serum CTX-1, these findings indicate the lower cancellous bone volume fraction and reduced bone formation in rapamycin-treated mice may be influenced by multiple mechanisms including locally reduced osteoblast activity and increased osteoclast activity. Our in vivo results in mice are consistent with several studies demonstrating that rapamycin or genetic inhibition of mTOR suppresses osteoblast activity and enhances osteoclastogenesis in vitro \cite{12,17,18,43}.

It is hypothesized that rapamycin influences healthspan and lifespan, in part, through inhibition of inflammatory signaling pathways, thus reducing tissue-level and systemic inflammation. Our group has previously demonstrated that rapamycin treatment attenuates inflammatory gene expression in lung and white adipose tissue in mice \cite{44,45}, and other groups have shown similar effects in other tissues. In the present study, we observed no impact of rapamycin on gene expression of prototypical inflammatory chemokines and cytokines in homogenized whole tibia, which is comprised of both bone marrow and bone. Additionally, we also found no rapamycin-induced reduction in inflammatory markers in tibia from Nrf2\textsuperscript{−/−} mice, which had significantly elevated inflammation. The exact reason bone and/or bone marrow is refractory to rapamycin-induced alteration in inflammatory status is unclear.

![Experimental Gerontology 154 (2021) 111516](https://example.com/image.png)
However, there is a major translational issue with studying aging-related metabolic bone disease in mice, in that the temporal pattern of bone loss between mice and humans is markedly different. In mice, cancellous bone mass peaks well before skeletal maturity which occurs at ~6 months of age; therefore, in aged animals, the majority of their cancellous bone at some clinically relevant skeletal sites (e.g., distal femur) has been resorbed, and little cancellous bone is available to effectively model interventions to attenuate aging-related skeletal impairment [4,35]. In contrast, human cancellous bone loss occurs long after skeletal maturity. While this premature bone loss in mice is strongly associated with cold temperature stress induced by housing mice below their thermoneutral zone, the vast majority of aging studies with mice were performed at room temperature, a trend unlikely to change [4]. Thus, it is important to study younger mice and experimental mouse models of accelerated aging, such as Nr2f2−/− mice. Additional work will be required to directly test whether rapamycin exerts differential effects on the skeleton throughout skeletal development and maturation, maintenance, and senescence.

We utilized Nr2f2−/− mice to determine whether rapamycin exerts protective effects in an experimental model of oxidative damage and aging-related disease vulnerability. Nr2f2 is a central regulator of the antioxidant response and accordingly, it has been reported that Nr2f2−/− mice have significantly elevated levels of oxidative stress and inflammation across multiple tissues [29–33]. Here, we demonstrate the inflammatory status of the skeleton is also sensitive to Nr2f2 levels, with Nr2f2−/− mice exhibiting significantly higher levels of tibia inflammatory gene expression compared to WT mice. Despite these robust genotype differences in skeletal inflammatory status, we observed no differences in skeletal phenotype between WT and Nr2f2−/− mice, which is surprising given the detrimental role of ROS and inflammation on bone homeostasis. Our findings contrast with several reports of skeletal impairment in Nr2f2−/− mice [46–49]; however, they do agree with those of Pellegrini et al. who examined bone mineral density from 1 to 15 months-of-age in female and male WT and Nr2f2−/− mice [50]. While these researchers observed both significant sex- and age-specific effects of Nr2f2 deletion on bone accrual and metabolism, they found minimal differences in BMD between female WT and Nr2f2−/− mice at 7 months-of-age, which is the approximate age of the mice in our study. Our findings are also in agreement with a long-term spaceflight study demonstrating that spaceflight significantly influenced the Nr2l2 signaling pathway and mimicked aging-like changes of plasma metabolites and tissue gene expression in mice, but Nr2f2 deficiency had minimal impact on the progression of spaceflight-induced skeletal impairment [39,51]. Altogether, these findings suggest that the association between Nr2f2 levels and bone metabolism is situation dependent.

The present study has several limitations. As previously mentioned, the study focused on a time-point at which the mice were reaching skeletal maturity, and the findings may not be applicable to rapamycin treatment initiated in older rodents. We performed the study in females; however, numerous lines of evidence indicate a sexually dimorphic response to rapamycin treatment, with a greater geroprotective benefit typically observed in female mice compared to male mice. We also did not assess mTORC1 and mTORC2 inhibition status in the bone or bone marrow, and it is possible our rapamycin dosage and/or dosing schedule inhibited both complexes. Given the large body of evidence demonstrating negative side effects of rapamycin treatment are due to inhibition of mTORC2, it is possible the observed adverse effects of rapamycin on the skeleton could also be due to direct mTORC2 inhibition in bone cells. This aligns with work from Chen et al. demonstrating deletion of Rictor, a critical component of mTORC2, in the skeleton of mice resulted in impaired bone formation [11]. Alternatively, the observed rapamycin-induced skeletal impairment could be a secondary or indirect effect of mTORC2 inhibition in another tissue. For example, the rapamycin dosage and dosing schedule used in this study has been demonstrated to cause insulin resistance via inhibition of liver mTORC2 [52–54]; insulin resistance is associated with bone loss in mice, and it is possible the observed skeletal impairment may be a downstream effect of rapamycin-induced systemic metabolic dysfunction. Further investigation is warranted to determine if the results from this study are applicable to male mice and to determine if local or global mTORC2 inhibition may be mediating the observed effects on the skeleton. Finally, rodents are the most widely used experimental model for bone growth and turnover, but there are species-specific differences in bone physiology and metabolic regulation that may preclude direct translation of rodent findings to humans.

In summary, we demonstrate that treatment of young-adult female WT and Nr2f2−/− mice with rapamycin resulted in lower femur bone mass and alterations in the cancellous bone microarchitecture and volume in the distal femur metaphysis and lumbar vertebra compared to placebo-treated mice. These differences occurred coincidentally with lower bone formation rate and higher circulating CTX-1 in rapamycin-treated mice. Establishing the overall impact of rapamycin treatment, as well as the nuances of rapamycin treatment (i.e., dosage, dosing schedule, route of delivery, age of initiation) on skeletal physiology across different experimental models and throughout lifespan will allow researchers to better appreciate its clinical potential as an anti-aging therapy.

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Author contributions

V.I.P. designed the research; S.A.M., R.T.R., R.W., Z.Y., A.M.A-B, C.P.W., and D.A.O. performed the research; A.J.B analyzed the data; S.A.M., R.T.T., and U.T.I. wrote the manuscript. All authors edited the manuscript and approved the final version.

Declaration of competing interest

The authors declare no competing interests.

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