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Research Article

Dexamethasone-induced muscle atrophy and bone loss in six genetically diverse collaborative cross founder strains demonstrates phenotypic variability by Rg3 treatment

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ABSTRACT

Background: Osteosarcopenia is a common condition characterized by the loss of both bone and muscle mass, which can lead to an increased risk of fractures and disability in older adults. The study aimed to elucidate the response of various mouse strains to treatment with Rg3, one of the leading ginsenosides, on musculoskeletal traits and immune function, and their correlation.

Methods: Six Collaborative Cross (CC) founder strains induced muscle atrophy and bone loss with dexamethasone (15 mg/kg) treatment for 1 month, and half of the mice for each strain were orally administered Rg3 (20 mg/kg). Different responses were observed depending on genetic background and Rg3 treatment.

Results: Rg3 significantly increased grip strength, running performance, and expression of muscle and bone health-related genes in a two-way analysis of variance considering the genetic backgrounds and Rg3 treatment. Significant improvements in grip strength, running performance, bone area, and muscle mass, and the increased gene expression were observed in specific strains of PWK/PhJ. For traits related to muscle, bone, and immune functions, significant correlations between traits were confirmed following Rg3 administration compared with control mice. The phenotyping analysis was compiled into a public web resource called Rg3-OsteoSarco.

Conclusion: This highlights the complex interplay between genetic determinants, pathogenesis of muscle atrophy and bone loss, and phytochemical bioactivity and the need to move away from single inbred mouse models to improve their translatability to genetically diverse humans. Rg3-OsteoSarco highlights the use of CC founder strains as a valuable tool in the field of personalized nutrition.

1. Introduction

Osteosarcopenia is a term used to describe the co-occurrence of osteoporosis (a disease characterized by low bone density and bone tissue deterioration) and sarcopenia (age-related loss of muscle mass and strength). The exact prevalence of osteosarcopenia is not well defined; however, it is estimated that the incidence rate of osteoporosis and sarcopenia increases with age, affecting millions of individuals worldwide, especially older adults. Management of osteosarcopenia involves a combination of lifestyle modifications, pharmacotherapy, and surgery in severe cases [1,2]. Immunological dysregulation and chronic inflammation have been discussed in the multifaceted pathogenesis of osteosarcopenia. Recent studies have shown that skeletal muscle regulates immunological processes and inflammatory responses [3]. The regenerative potential of skeletal muscle depends on the interaction between skeletal muscle and immune cells, and as people age, the

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physiological functions of immune cells are gradually lost, reducing the regenerative ability of skeletal muscle [4].

There are many causes of osteosarcopenia, but glucocorticoid excess is the second most common cause of muscle atrophy and bone loss after aging [5]. Immunosuppressive therapy with glucocorticoids is highly beneficial for a wide range of inflammatory diseases. However, glucocorticoid therapy has a devastating effect on the musculoskeletal system, with muscle atrophy and bone loss occurring in approximately 30–50 % of patients who receive long-term glucocorticoid therapy [5,6]. Glucocorticoids increase bone fragility by inducing bone loss, decreasing bone formation, and thinning trabecular and cortical bone. Glucocorticoids also induce a decrease in skeletal muscle mass and muscle weakness, particularly in the hip and shoulder girdle muscles, which increases the risk of falls. The combined effects on bone and muscle are the main reasons for the increased risk of fracture from glucocorticoid excess.

Phytochemicals are various types of secondary plant metabolites that have been reported to enhance various physiological activities in the human body [7,8]. Several studies have suggested the potential protective effects of phytochemicals against muscle atrophy and bone loss [9,10]. *Panax ginseng* is a plant that has been traditionally used in Asian medicine and has various health benefits, including beneficial effects on bone health, muscle strength, and immune function [11–14]. Ginsenoside Rg3, an active compound found in *Panax ginseng*, has been studied for its effects on osteoporosis, a condition characterized by low bone mass, decreased bone strength, and osteosarcopenia, which refers to the loss of both muscle mass and bone density [15–17]. Treatment with Rg3 improves bone mass, reduces bone resorption markers in ovariectomized mice, increases the proliferation of myoblasts and muscle function, and suppresses T lymphocytes [18–21]. This suggests that Rg3 may have beneficial effects on immune function and can potentially be used for the treatment of muscle atrophy and bone loss.

Recently, several claims have been made regarding the disconnection between preclinical and clinical studies of phytochemicals. These include the use of unreasonably high untranslatable doses in preclinical models that do not account for the doses administered to humans. Another limitation is the lack of translatability in mouse models. Although several mouse strains are available, most studies on the phytochemicals used to treat muscle atrophy and bone loss have been conducted in the context of immobilization or chemical induction in several mouse strains, particularly in male C57BL/6J (B6) mice [22–24]. Reliable responses from phenotyping of a single strain are only suitable for phenotypes or diseases in which the interactions between specific genetic factors and traits are not considered. Male B6 mice have been extensively used to evaluate the potential benefits of phytochemicals with respect to muscle atrophy and bone loss; however, few phenotypic data have been studied regarding the response of different genetic backgrounds to phytochemicals. The magnitude, direction, and consistency of responses to phytochemicals can greatly vary between the genetic backgrounds of mouse models; however, these observations have rarely been studied. Therefore, studies on the effects of phytochemicals on muscle atrophy and bone loss in more complex and genetically diverse animals are required. Phytochemicals have distinct effects on specific traits in different mouse strains. For example, glutathione homeostasis in female CAST mice is regulated by the administration of cyanidin-3-*O*-glucoside, whereas other strains are non-responsive (NOD/ShiLtJ strain) or barely respond (A/J, 129S1/SvImJ, and B6 strains) to the administration of cyanidin-3-*O*-glucoside [25]. In trinitrobenzene sulfonic acid-induced colitis, curcumin increases survival, reduces weight loss, and attenuates disease progression in BALB/c mice, but not in SJL/J mice [26].

A large panel of reproducible recombinant inbred mouse strains, the Collaborative Cross (CC) mouse model, is a diverse inbred mouse model that can be used to discover disease targets and study treatment responses. CC strains are derived from eight genetically diverse founder strains, including inbred strains, such as B6 mice, and three wild inbred strains that contain ~90 % of the existing mouse genetic diversity [27]. Models of CC and CC founder strains can be useful for determining the translatability of phytochemicals in humans in mouse studies. To date, limited efforts have been made to investigate the effects of genetic factors on the pharmacological effects of natural products. One study reported that the rate of liver toxicity in response to green tea catechins in Diversity Outbred (DO) mice generated by random mating of CC strains, was similar to that in humans experiencing toxicity in human clinical trials [28]. Efforts utilizing these genetically diverse mouse models will ultimately pave the way for more targeted approaches that utilize phytochemicals for disease prevention and translation into genetically diverse human populations.

This study aimed to explore the response of genetically diverse mice to dexamethasone (DEX)-induced muscle atrophy and bone loss and subsequent treatment with Rg3 using the web-based resource, Rg3-OsteoSarco (https://myungsukkim.shinyapps.io/Rg3-OsteoSarco/). To the best of our knowledge, this is the first attempt to use the CC founder strains to study the effects of phytochemicals on osteosarcopenia. Given the well-documented effects of DEX and the preventive effect of Rg3 on muscle atrophy and bone loss in animal models, Rg3 was selected as a representative phytochemical to evaluate the effect of the genetic background on the ameliorative effects of phytochemicals on muscle atrophy and bone loss. We hypothesized that diverse mouse strains would respond differently to Rg3 treatment, suggesting a role for genetic factors in muscle atrophy and bone loss and opening the door to new genetic models that can help better understand the potential benefits of Rg3 treatment.

2. Materials and methods

2.1. Animals

Animal experiment was approved by the International Animal Care Committee of the Korea Institute of Science and Technology (approval number: KIST-2021-12-153). Eight 5-week-old male mice per strain were purchased from The Jackson Laboratory (Bar Harbor, ME): A/J (stock number 000646), C57BL/6J (000664), 129S1/SvImJ (002448), NOD/ShiLtJ (001976), NZO/EiJ (002105), and PWK/PhJ (003715). After 2 weeks of acclimatization, half of them were divided into the control group and the other half into the Rg3 group. All cages were maintained in identical conditions maintained at constant temperature (21–22 °C) and humidity (55 ± 5 %) conditions with a standard 12:12 light/dark cycle. Rg3 (20 mg/kg/day, ChemFaces, Wuhan, Hubei, China) was dissolved in a 0.5 % vehicle solution of sodium carboxymethyl cellulose (CMC) (Sigma Aldrich, St. Louis, Missouri, US) and then orally administered daily for 6 weeks. A control group for each strain was treated with vehicle solution only. After oral administration for the first 2 weeks, all mice were intraperitoneally injected with DEX (15 mg/kg/day). Many studies have reported that 2 weeks to 1 month of DEX treatment results in muscle atrophy or bone loss in mice and rats [29–35]. We induced both muscle atrophy and bone loss by intraperitoneally injecting DEX for 1 month. Establishing a DEX-free control group for all mouse strains was ideal, but sample size and cost constraints made it prohibitive. Animals were allowed access to food and water ad libitum.

After 6 weeks of oral administration of Rg3, mice were euthanized and sacrificed after 16 h fasting. Plasma and muscle (quadricep, gastrocnemius, tibialis, and soleus muscles), bone (femurs), and spleen samples were collected from each mouse and stored at −80 °C for further study. Muscle tissue was quickly weighed on an analytical balance (MS204, Mettler Toledo, Switzerland) after dissect and frozen in liquid nitrogen. The rate of muscle atrophy was calculated as the ratio of muscle weight to body weight.

2.2. Grip strength test and treadmill running exercise

Grip force was measured using a grip force meter (Bioseb, Pinellas Park, FL, USA). The mouse grabbed the grid with its forelimbs and pulled gently in the opposite direction with constant force until the forelimbs detached from the grid. Three trials of three trials were performed for each mouse, for a total of nine. A Touchscreen Treadmill (Panlab, Harvard Apparatus, Holliston, MA, USA) was used to test the endurance of the mouse. Prior to testing, mice were acclimated to the treadmill running for 15 min at 10 m/min. The treadmill test was started at 10 m/min and increased to 1 m/min per minute until the mouse was exhausted. During running, the mouse was prevented from resting by a

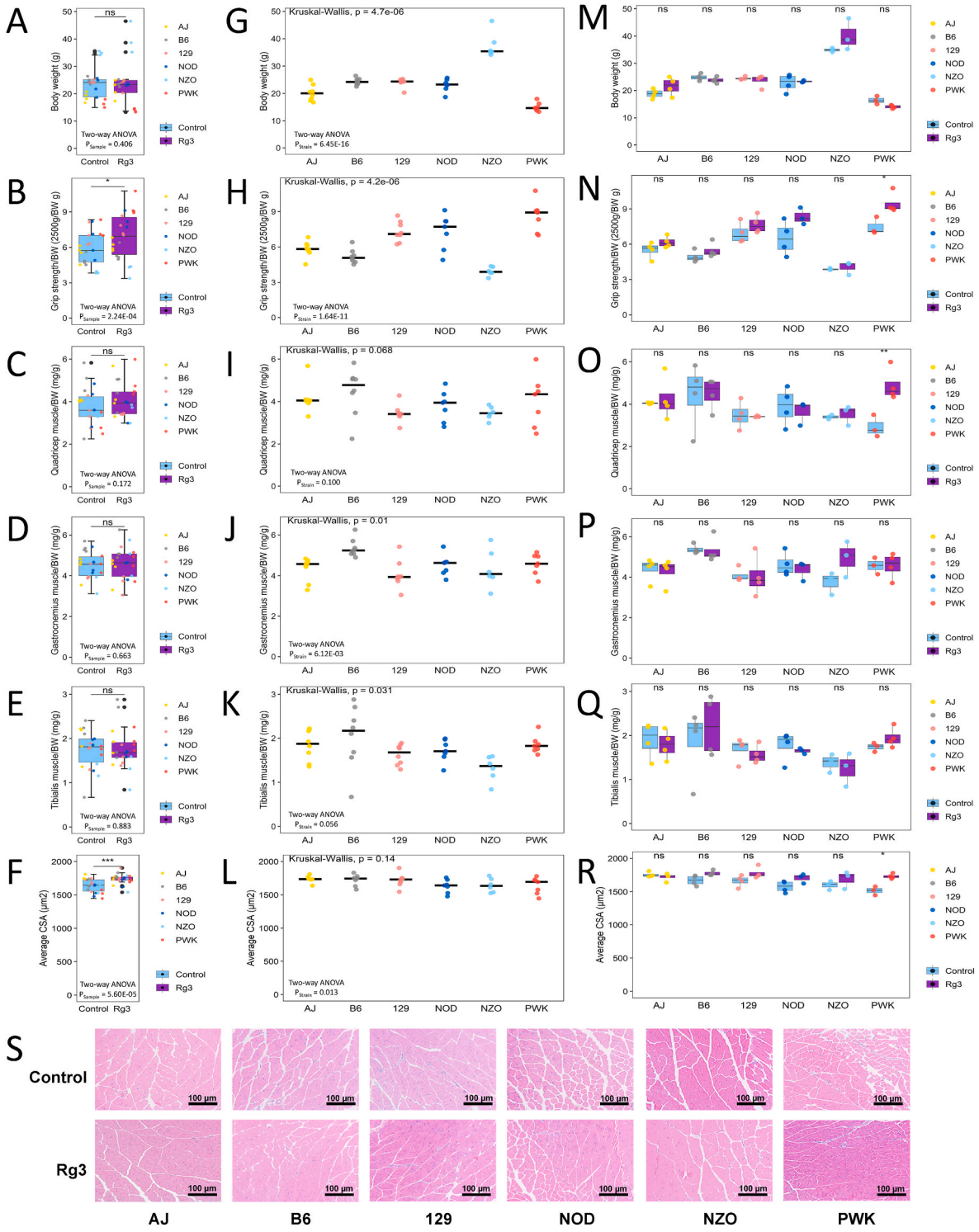

Fig. 1. Rg3 or strain-dependent differences in body weight, grip strength, muscle mass, and muscle fiber in six CC founder strains. (A–R) Rg3 effects in all groups (A–F), strain effects (G–L), and Rg3 effects in each strain (M–R) were assessed for body weight, grip strength, three types of muscles (quadricep, gastrocnemius, and tibialis muscles) normalized to body weight, and muscle fiber size. Comparisons between control and Rg3 groups were performed by Wilcoxon test and between strains by Kruskal-Wallis test. Data were mean ± S.E., n ≥ 3–4 mice/sample/strain. (S) Comparison of the size of H&E stained muscle fibers in the gastrocnemius muscle between the control and Rg3 groups in each strain. The magnitude was 100×.

shock bar placed behind the treadmill set at 20 V. Calculation of running distance and workload was completed when the mouse stopped moving for >10 s. Distance traveled was calculated as the product of travel time and speed.

2.3. Biochemical analysis in plasma

To assess muscle damage, lactate dehydrogenase (LDH) levels in the plasma were evaluated according to manufacturer specifications (abcam, Cambridge, UK).

2.4. Histological analysis of skeletal muscle

Gastrocnemius muscle samples were fixed in 4 % formalin overnight, then embedded in paraffin, and serially sliced to 4 μm for Hematoxylin and eosin (H&E) staining. The ratio of muscle fiber area to total cross-sectional area was measured using the ImageJ software [36]. The diameters of all muscle fibers in each view (100 × objective, ZEISS, Oberkochen, Germany) were calculated using ImageJ software and marked as a percentage of the control.

2.5. Data analysis and statistics

Data were analyzed using R (v.4.1.2) (R Core Team). Differences between the control and Rg3 groups were evaluated by the Wilcoxon test. Differences in each strain were assessed by Kruskal-Wallis test. A two-way ANOVA was performed to determine the significance of the main effect (sample effect) and interaction (strain * sample) for each stage ($\alpha = 0.05$) for all mice in each strain. This analysis was performed to differentiate the effect of genetic background on the effects of Rg3 treatment on muscle and bone health-related traits. Spearman's correlation was used to correlate the traits and microbial taxa abundance. The p-values were adjusted using the BH false discovery rate (FDR) procedure [37], and correlation coefficients and adjusted p-value were visualized using the 'pheatmap' package [38].

2.6. Supporting information

Details of materials preparation and full experimental procedures, including histological analysis of femurs, measurement of ATP content in muscle, measurement of enzyme activation in muscle, flow cytometry analysis, quantitative real-time polymerase chain reaction (qRT-PCR) analysis are given in the Supplementary Information.

3. Results

3.1. Effect of genetic backgrounds and Rg3 on grip strength, muscle mass, and myofiber size

To investigate the differences in severity of muscle atrophy in each strain and to determine whether Rg3 treatment increased muscle function and mass, grip strength, muscle weight, and muscle fiber area were assessed (Fig. 1). In addition, we created Rg3-OsteoSarco (https://myungsukkim.shinyapps.io/Rg3-OsteoSarco/), a web-based resource that makes strain-specific Rg3 responses and trait–trait correlations accessible to the public. Rg3-OsteoSarco allows users to search for sample or strain-specific muscle and bone health-related traits and trait–trait correlations. In the two-way analysis of variance (ANOVA) considering the interaction between the two groups (control and Rg3) and strains, Rg3 significantly increased grip strength ($P = 2.24 \times 10^{-4}$) and average cross-sectional area of gastrocnemius myofibers ($P = 2.49 \times 10^{-4}$) in all strains (Fig. 1B and F). However, no significant difference was identified in body weight (Fig. 1A) and muscle weight (Fig. 1C–E and Table S1). We also found a significant genetic effect on grip strength in the two-way ANOVA (Fig. 1H and Table S1), and the median value of each strain was highest in the wild-derived strain PWK and lowest in the fattest NZO strain (Fig. 1H). In addition, there was a significant difference in gastrocnemius muscle weight between the strains (Fig. 1J and Table S1), and the median muscle weight was the highest in the B6 strain and the lowest in the NZO strain (Fig. 1I–K). The relatively low muscle weight of the NZO strain compared with other strains was confirmed in a similar strain survey study [39]. For the Rg3 effect on grip strength and muscle weight in each strain, Rg3 increased grip strength and quadriceps muscle weight only in the PWK strain (Fig. 1N and O). Strain-specific drug responsiveness in the PWK strain has also been reported in a previous study on weight loss following phytochemical administration [40]. H&E staining of the cross-sectional area of the muscle fibers revealed that significant strain effect was identified by the two-way ANOVA (Fig. 1L and Table S1) and that the fibers were densely packed within the same magnification and increased in PWK strain by Rg3 treatment (Fig. 1R and S and Table S2).

3.2. Effect of genetic backgrounds and Rg3 on exercise performance and biomarkers of mitochondrial biogenesis and muscle injury

Based on the differences in grip strength, muscle mass, and strain-specific drug responses between strains, we measured endurance-related traits, including exercise performance, mitochondrial function, and muscle damage markers (Fig. 2). First, in the two-way ANOVA considering the interaction between the sample and genetic effects, Rg3 significantly increased the distance to exhaustion ($P = 0.033$) and plasma lactate dehydrogenase (LDH) ($P = 0.013$) levels, and maximum running speed ($P = 0.089$) and time to exhaustion ($P = 0.089$) also slightly increased (Fig. 2A–F and Table S1). There were significant strain effects on exercise performance in two-way ANOVA (Table S1). Based on the median value of endurance-related traits in each strain, the NOD strain was the highest, whereas the AJ and 129 strains were the lowest (Fig. 2G–I). This trend was consistent with a behavioral test study using AJ, 129, NOD strains, and CC strains [41]. However, there were no significant differences between the strains in muscle ATP, thiobarbituric acid reactive substance (TBARS), aconitase, citrate synthase, and plasma LDH in the two-way ANOVA (Fig. 2J–L and Table S1). The Wilcoxon test was performed to investigate the effect of Rg3 on exercise performance, mitochondrial function, and muscle damage markers in each strain. Rg3 increased exercise performance and decreased the level of plasma LDH, a muscle damage marker, in NZO and PWK. Muscle ATP and aconitase levels tended to increase in some strains, but were not significant because of the lower number of mice per group (Fig. 2M–R).

3.3. Effect of genetic backgrounds and Rg3 on bone health-related traits

Next, we evaluated bone health-related markers to confirm the difference in traits-related to osteoporosis induced by the strain and determined whether bone loss was improved by Rg3 administration. As bone weight greatly varies depending on the strain, all bone health-related traits were normalized by body weight (Fig. 3). In the two-way ANOVA, Rg3 increased the bone area/bone volume in all strains ($P = 0.058$), but there were no significant differences in other bone traits (Fig. 3A–F and Table S1). In terms of the strain effect, all traits related to bone health showed strong significant differences based on the median value of each strain; the wild-derived strain PWK was the highest, and the 129 or NZO strain was the lowest (Fig. 3G–L and Table S1). This strain effect on bone health-related traits is consistent with muscle atrophy-related traits, and the relatively high bone volume and trabecular plate number of the PWK strain compared with other strains have recently been reported in other CC founder strains [42,43]. For Rg3 effects on bone health in each strain, Rg3 increased BMD, bone area/-bone volume, bone area/total volume, and trabecular thickness only in PWK strain (Fig. 3M–R). Considering that wild-derived strains, including the PWK strain, show significant differences in bone health depending on the presence or absence of immobilization [42], our results show that the PWK strain is suitable for the study of drug responses

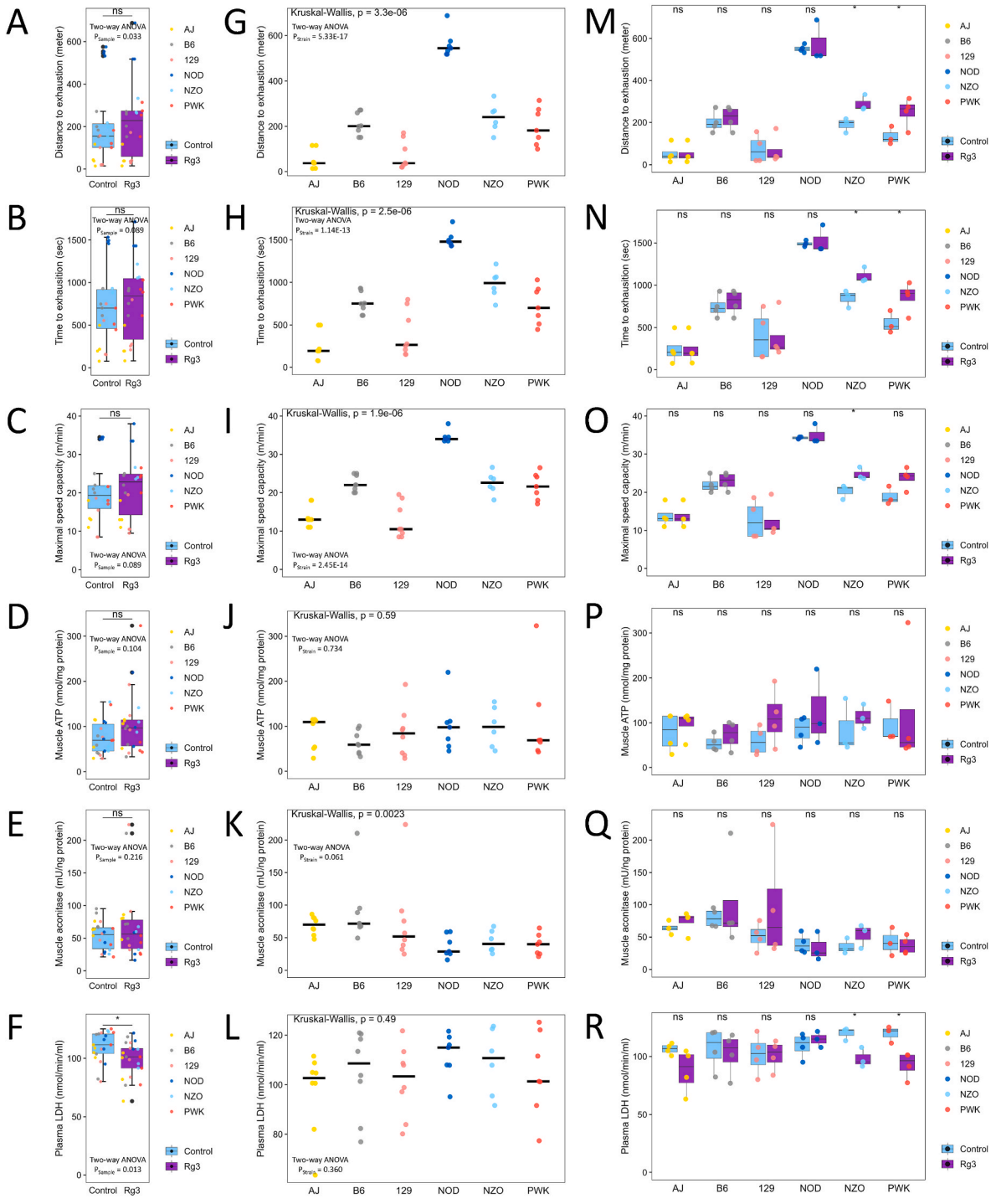

Fig. 2. Rg3 or strain-dependent differences in endurance, muscle ATP, mitochondrial function, and muscle damage marker in six CC founder strains. (A–R) Rg3 effects in all groups (A–F), strain effects (G–L), and Rg3 effects in each strain (M–R) were assessed for distance to exhaustion, time to exhaustion, maximal speed capacity, tibialis muscle ATP, tibialis muscle aconitase, and plasma LDH. Comparisons between control and Rg3 groups were performed by Wilcoxon test and between strains by Kruskal-Wallis test. Data were mean ± S.E., n ≥ 3–4 mice/sample/strain.

in muscle atrophy and bone loss.

3.4. The immune function of the spleen was not significantly affected by Rg3 treatment or genetic background

Recent studies have revealed a complex interplay between the skeletal muscle and immune system that regulates muscle regeneration [44, 45]. The contribution of T cells to muscle injury and regeneration processes has been highlighted, as has the role of specific T cell subsets [46, 47]. Considering the association between muscle atrophy and bone loss and immune function, we measured the levels of $CD4^+$ and $CD8^+$ T cells and inflammatory cytokines in the spleen. No significant differences were observed in either the samples or strains (Fig. S1G–L and Table S1). Regarding the effect of Rg3 on immune function in each strain, Rg3 showed a slight increase or decrease in some traits in each strain, but these changes were not significant overall (Fig. S1M–R). These results suggest that the effect of the strain or Rg3 on immune function traits is minimal in the DEX-induced CC founder strains.

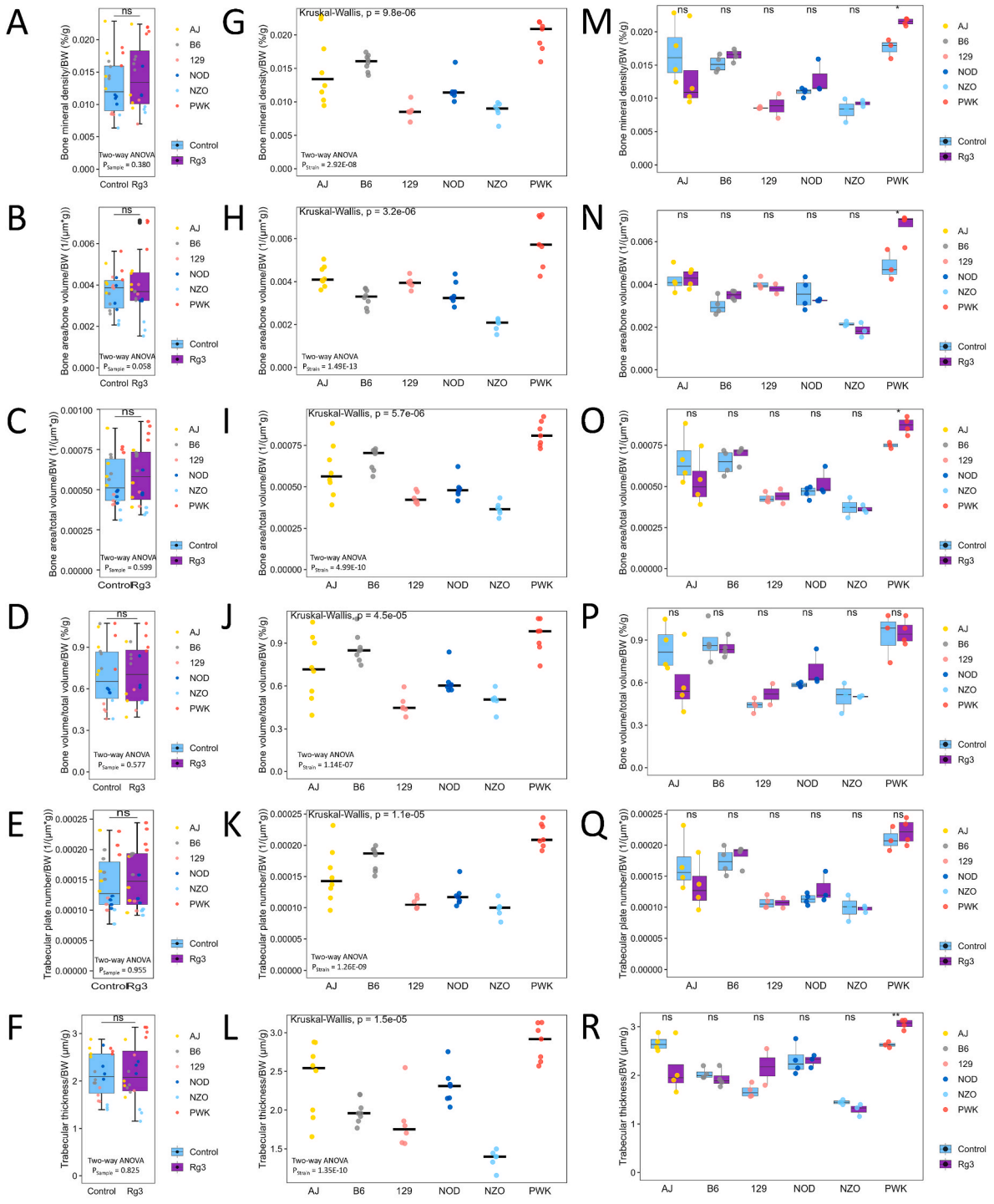

Fig. 3. Rg3 or strain-dependent differences in bone heath traits in six CC founder strains. (A–R) Rg3 effects in all groups (A–F), strain effects (G–L), and Rg3 effects in each strain (M–R) were assessed for bone mineral density, bone area/bone volume, bone area/total volume, bone volume/total volume, trabecular plate number, and trabecular thickness. All bone traits were normalized by body weight. Comparisons between control and Rg3 groups were performed by Wilcoxon test and between strains by Kruskal-Wallis test. Data were mean ± S.E., n ≥ 3–4 mice/sample/strain.

3.5. Rg3 strengthened the association between musculoskeletal traits and immune function

Next, we investigated whether the muscle and bone health-related traits measured in the muscle, bone, and spleen were correlated with each other and whether the degree of these correlations varied with Rg3 treatment (Fig. 4). Interestingly, some muscle and bone health-related traits showed significant correlation regardless of Rg3 treatment, and the correlation between these traits became prominent by Rg3 treatment. For example, weight was negatively correlated with grip strength and bone traits, whereas soleus muscle weight was positively correlated with bone traits (Fig. 4A lower panel). This association has been verified in humans and in various rodent models [48–50]. Additionally, Rg3 treatment further enhanced this correlation (Fig. 4A and B). Only in the Rg3 group, endurance performance was negatively correlated with bone area/bone volume and muscle TBARS (Fig. 4A and C), and gastrocnemius muscle weight was negatively correlated with tumor necrosis factor (TNF)-α, an inflammatory cytokine in $CD4^+$ T cells

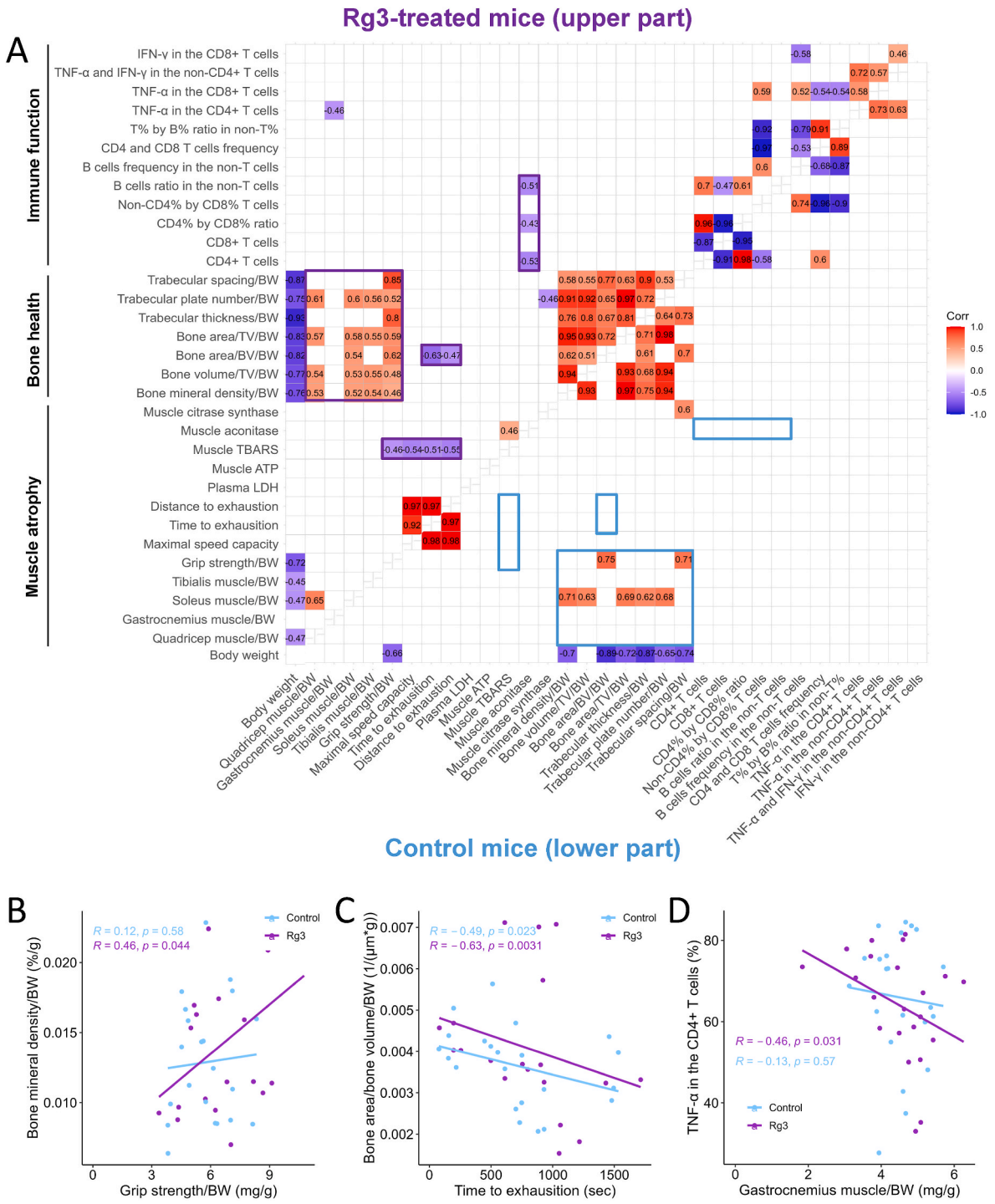

Fig. 4. Correlation between muscle atrophy and bone loss-related traits in six CC founder strains. (A) Spearman correlation between muscle atrophy and bone loss-related traits in control group (lower panel, lightblue color) or Rg3-treated group (upper panel, purple color). The p-values were adjusted using the BH FDR procedure. Only correlations with an adjusted p-value <0.05 shown in the heatmap. (B) Spearman correlation between grip strength and bone mineral density in each control and Rg3 group. (C) Spearman correlation between time to exhaustion and bone area/bone volume in each control and Rg3 group. (D) Spearman correlation between TNF-α in the $CD4^+$ T cells and gastrocnemius muscle in each control and Rg3 group.

(Fig. 4D). These results are consistent with those of previous studies demonstrating that physical activity enhances antioxidant defense and lowers lipid peroxidation levels in both adults and the elderly [51–53]. Thus, this study recapitulates the association between muscle and bone health-related traits identified in humans and CC founder strains with diverse genetic backgrounds.

3.6. Effect of genetic backgrounds and Rg3 on the expression of key muscle and bone-related genes and trait-gene correlation

Based on the correlation results of traits related to muscle strength and endurance with bone health and immune functions, we investigated the differences in the expression of genes related to muscle strength and endurance between strains or samples and determined whether these genes were correlated with muscle and bone health-related traits

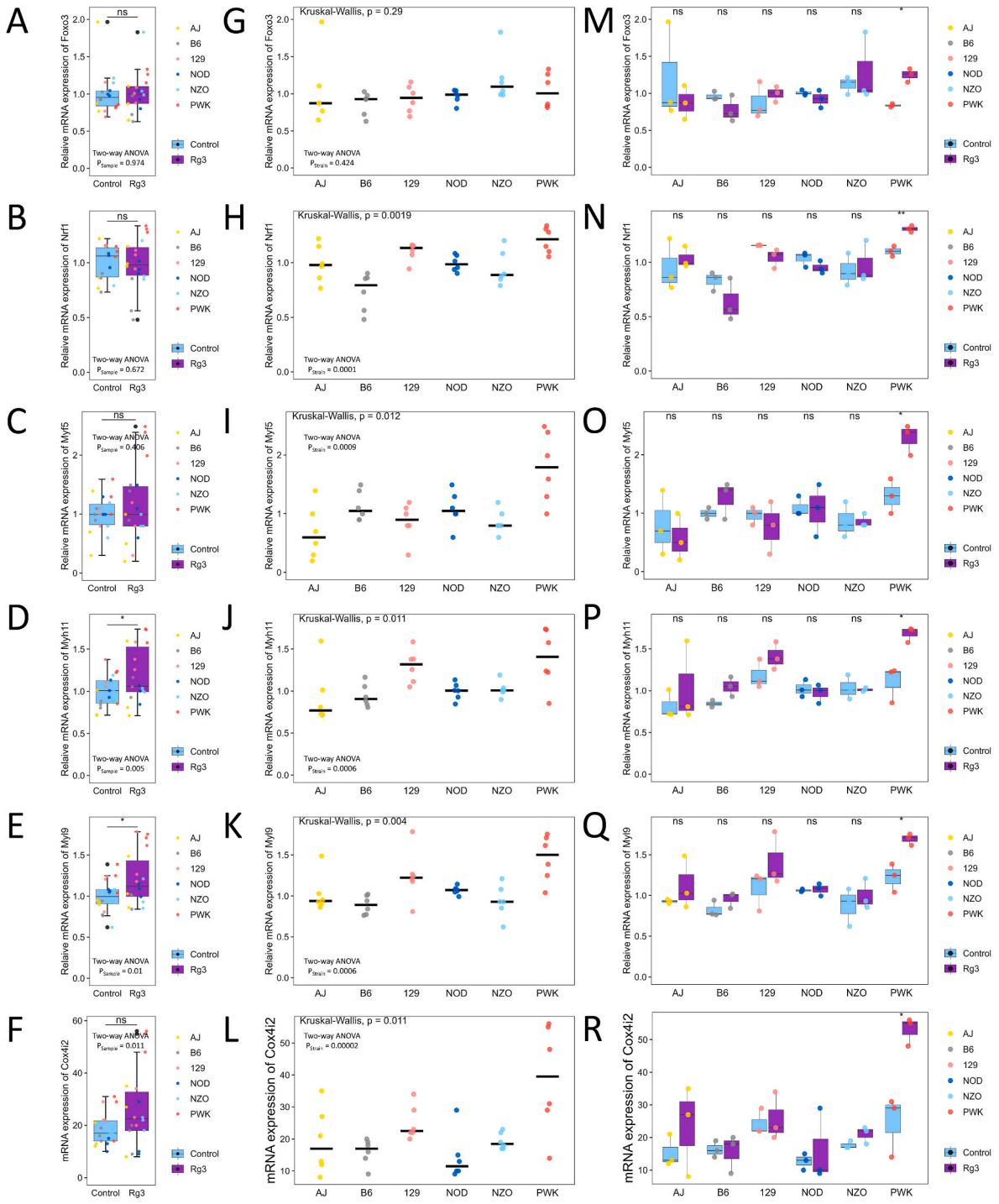

Fig. 5. Rg3 or strain-dependent differences in mRNA expressions of muscle function-related genes in six CC founder strains. (A–R) Rg3 effects in all groups (A–F), strain effects (G–L), and Rg3 effects in each strain (M–R) were assessed for mRNA expression of *Foxo3*, *Nrf1*, *Myf5*, *Myh11*, *Myl9*, and *Cox4i2*. Comparisons between control and Rg3 groups were performed by Wilcoxon test and between strains by Kruskal-Wallis test. Data were mean ± S.E., n ≥ 3–4 mice/sample/strain.

(Fig. 5). We measured the mRNA expression of 33 genes previously related to muscle strength and endurance in the tibialis muscle, and some of them showed significant strain or sample effects. In particular, the upregulation of gene expressions by Rg3 was remarkable in the PWK strain. For example, the expressions of *Foxo3* and *Nrf1*, transcription factors that regulate the homeostasis of the skeletal muscle and mitochondrial function, increased by Rg3 in the PWK strain [54–56] (Fig. 5A–N). In addition, the expressions of *Myf5*, which is a myogenic regulatory factor; *Myh11*, which is the isoform of the myosin heavy chain; *Myl9*, which is the isoform of the myosin light chain; and *Cox4i2*, which is the complex IV subunit associated with mitochondrial function, were highest in the PWK strain and increased by Rg3 treatment (Fig. 5C–R). These results suggest that the PWK strain is suitable for the study of muscle and bone health-related traits and for pharmacological testing of phytochemicals by maintaining high expression of key genes that regulate muscle strength and endurance.

Finally, we investigated whether the expression levels of the genes measured in the tibialis muscle tissues were correlated with muscle and bone health-related traits (Fig. 6). *Myog*, which is a myogenic regulatory factor, *Myh7*, and *Myl12a* have strong positive correlations with grip

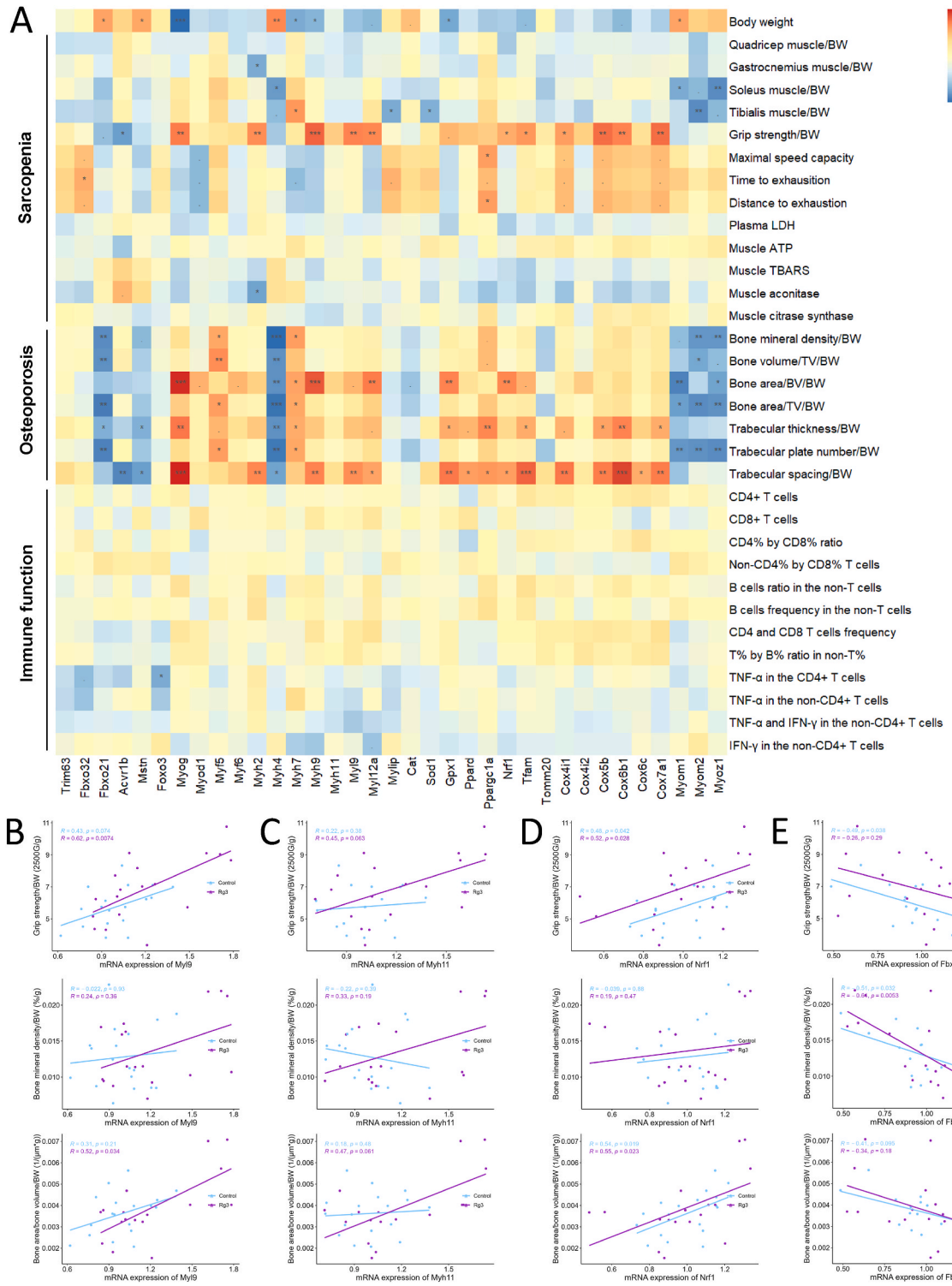

Fig. 6. Correlation between muscle atrophy and bone loss-related traits and key muscle function-related genes in six CC founder strains. (A) Spearman correlation between muscle atrophy and bone loss-related traits and key muscle and bone function-related genes in all mice. The p-values were adjusted using the BH FDR procedure. "***" $P < 0.001$, "**" $P < 0.01$, and "*" $P < 0.05$. (B–E) Spearman correlation between grip strength, bone mineral density, or bone area/bone volume and four candidate genes expression including *Myl9* (B), *Myh11* (C), *Nrf1* (D), *Fbxo21* (E) in each control and Rg3 group.

strength, bone area, and trabecular thickness, and *Ppargc1a*, a transcription factor that regulates mitochondrial genes, has a positive correlation with endurance traits (Fig. 6A). Interestingly, these correlations were more prominent in the Rg3 group than in the control group. For example, *Myl9*, *Myh11*, and *Nrf1* showed stronger correlations with grip strength and bone area/volume in the Rg3 group (Fig. 6B–D). On the other hand, *Fbxo21*, which is associated with muscle atrophy and bone loss [57,58], was negatively correlated with grip strength and bone mineral density (Fig. 6E). These results show that regardless of the genetic background, key genes regulating muscle atrophy and bone loss are associated with muscle and bone health-related traits and that Rg3 mediates the association between these traits.

4. Discussion

In this study, we investigated how Rg3, a major component of red ginseng, affects muscle, bone health, and immune function-related phenotypes in diverse mouse genetic backgrounds using phenotyping tools and the results archived on the public web resource Rg3-OsteoSarco. This was a preliminary study to utilize future cohorts of CC or DO mice from a nutrigenomics perspective. Several results were derived from this study. First, dramatic differences in muscle atrophy and bone loss were observed between the CC founder strains. In general, high muscle strength, BMD, and weight were observed in the B6 and PWK strains, whereas high endurance was observed in the NOD strain. Second, only some strains showed significant effects of Rg3 on muscle and bone health-related traits. The responsiveness of the muscle and bone health-related phenotype to Rg3 was highest in PWK. Third, significant correlations were observed between muscle, bone health, and immune function-related traits in the Rg3-treated and untreated groups. When treated with Rg3, BMD was positively correlated with grip strength, and TNF-α in $CD4^+$ T cells was negatively correlated with muscle mass. Finally, genes associated with muscle atrophy and bone loss were significantly correlated with grip strength and bone area in the CC founder strains. Each of these points is discussed below.

Among the phytochemicals, we focused on ginsenoside Rg3, which is a functional ingredient of Korea red ginseng [59,60] and mitigates muscle atrophy and bone loss in preclinical and clinical trials [16,61,62]. In addition, we used a DEX-induced model to effectively induce muscle atrophy and bone loss among several methods, including chemotherapy (cisplatin) [63], fasting [64], nephrectomy [65], and hindlimb unloading [66], based on literature survey and previous studies. In terms of the efficacy of Rg3 on muscle atrophy-related traits, there were significant differences in the effects of Rg3 between PWK and DEX-induced CC strains. Administration of Rg3 significantly increased grip strength and quadriceps muscle strength in the PWK strain, and two-way ANOVA confirmed the increase in grip strength in all strains. The effect of Rg3 on endurance-related traits was only observed in the NZO and PWK strains, and these traits showed suggestive ($P < 0.1$) or significant ($P < 0.05$) differences in the two-way ANOVA. Notably, in the most obese NZO strain, Rg3 administration increased tibialis muscle ATP and aconitase levels along with exercise capacity (albeit not significantly) and decreased level of plasma LDH, a marker of muscle damage. Similar to the muscle atrophy-related traits, the effect of Rg3 on bone health was only observed in the PWK strain. The PWK strain also maintained high levels of muscle and bone health-related traits, including grip strength, muscle weight, BMD, bone area, and trabecular thickness. This finding is consistent with those of previous reports showing that bone loss is accompanied by muscle atrophy [67]. Recent studies have shown that conventional $CD8^+$ and $CD4^+$ T cells play an important role in muscle regeneration. For example, loss or gain of $CD8^+$ or $CD4^+$ T cells robs and restores muscle regenerative capacity, respectively [68,69]. Additionally, low CD4/CD8 T cell ratio is associated with aging and mortality [70], and Rg3 has been reported to increase the CD4/CD8 T cell ratio in the peripheral blood of cancer patients [71]. Our results showed that there was no significant effect of Rg3 and genetic background on $CD8^+$ and $CD4^+$ T cells isolated from the spleen and the cytokines secreted therefrom. In the NZO and PWK strains, Rg3 treatment increased the number of $CD4^+$ T cells and decreased the number of $CD8^+$ T cells, whereas the opposite trend was observed in the 129 and NOD strains (not significant). A decrease in TNF-α was identified in $CD4^+$ and non-$CD4^+$ cells in the Rg3-treated PWK strain. This reduction in inflammatory cytokines by Rg3 treatment may have resulted in an improvement in overall muscle and bone health-related traits.

Osteoporosis and sarcopenia commonly accompany aging, and as sarcopenia increases, the prevalence and mortality of osteoporosis also increase significantly [72]. For example, in a study of postmenopausal women, women with sarcopenia had up to a 12-fold increased risk of osteoporosis compared to women without sarcopenia [73,74]. Conversely, several studies have suggested that osteoporosis may increase the risk of sarcopenia [75,76]. Sarcopenia and osteoporosis are interacting musculoskeletal diseases, and both are associated with similar risk factors such as aging, gender, height, smoking, physical activity, lifestyle factors, and blood vitamin D levels, genetics, common pathogenic pathways, and endocrine function [74,77] Therefore, certain factors may mediate this bidirectional bone-muscle disturbance. In terms of molecular mechanisms, osteoporosis and sarcopenia share many common pathways, including decreased secretion of anabolic hormones, increased inflammatory cytokine activity, sensitivity to anabolic or catabolic molecules released by skeletal muscle or bone cells, and eventually reduced physical activity [78,79]. Underlying these factors is the immune system, which is an important source of cytokines and other secreted factors, including immune cells, suggesting that it may influence myogenesis and bone health [80,81]. In this study, we investigated whether there was a correlation between musculoskeletal traits and immune function and whether the degree of association was influenced by Rg3 administration. In general, grip strength and muscle weight showed a positive correlation with bone health traits, such as BMD and bone area, and endurance showed a negative correlation with bone area, which is consistent with the results of previous human and *in vivo* studies [82–84]. Additionally, we found that TNF-α, an inflammatory cytokine in $CD4^+$ T cells, was negatively correlated with gastrocnemius muscle, suggesting that an increase in muscle weight may suppress systemic inflammation. Interestingly, these trait–trait correlations were more pronounced in the Rg3-treated group than in the control group. For example, grip strength and muscle mass were significantly and positively correlated with BMD, bone volume, and bone area in the Rg3-treated group. TBARS, a lipid peroxidation marker, was negatively correlated with endurance, and aconitase activity, which is related to mitochondrial function, was negatively correlated with the population of $CD4^+$ T cells only in the Rg3-treated group. These results indicate that the administration of phytochemicals, including Rg3, may improve muscle atrophy and bone loss by strengthening the association between musculoskeletal traits and immune function. However, unlike the reported proportional relationship between grip strength and muscle mass, grip strength was not significantly correlated with any of the four muscle types in this study (Figs. S2A–S2D). Several studies have reported that the correlation between grip strength and muscle mass is higher in men than in women and in the elderly than in the young [85,86]. In our study, the diverse genetic background and relatively young age of 6 weeks, as well as the differential expression of muscle type-specific markers (e.g. *Myh2*, *Myh4*, and *Myh7*) between strains (Figs. S2H–S2J), may have reduced the correlation between grip strength and muscle mass.

Based on the strong correlations between musculoskeletal traits and similar strain responsiveness, we assessed the effect of Rg3 or genetic background on the expression of genes involved in proteolysis and bone loss, muscle cell differentiation, regeneration, and endurance. Similar to muscle atrophy and bone loss-related traits, the expression of most target genes was increased by Rg3 in the PWK strain (also confirmed by two-way ANOVA), especially the expression of myosins (*Myh2*, *Myh7*, *Myh11* and *Myl9*), which constitute muscle proteins, muscle cell differentiation marker (*Myf5*), and endurance markers (*Nrf1* and *Cox4i2*) were highest in PWK strain (Fig. S2). This suggests that the PWK strain not only has the highest proportion of both type 1 and type 2 fibers among all strains, but also that the expression of candidate genes is regulated by Rg3 treatment, resulting in increased grip strength, muscle mass, BMD, and bone area. Furthermore, trait–gene correlation analysis revealed that *Myl9*, *Myh11*, and *Nrf1* were positively correlated with grip strength and bone area, and these correlations were more pronounced by Rg3 administration (Fig. 6). *Fbxo21*, which is associated with muscle atrophy and bone loss [57,58], was negatively correlated with grip strength and bone area. However, *Trim63* (MuRF1), *Fbxo32* (Atrogin-1), and *Foxo3*, which are associated with the ubiquitin

proteasome system [87], did not show this negative correlation. This suggests that Rg3 alleviates muscle atrophy and bone loss by regulating the expression of specific genes in mouse models considering different genetic backgrounds.

To elucidate the underlying mechanisms linking genetic diversity to the effects of Rg3 on muscle atrophy and bone loss-related traits in different strains, characterizing genetic differences in muscle atrophy and bone loss-related traits between the six strains can be useful for identifying candidate genes and genetic variants that regulate responses to Rg3. The MGI and Wellcome Sanger Institute provide sequencing data of most of the inbred strains, including the eight CC founder strains. As a preliminary study, we analyzed the liver transcriptomes of eight CC founder strains fed a high-fat cholesterol diet and found that the NOX4, which is associated with hepatic triglyceride and plasma trimethylamine N-oxide level, may influence strain-specific responses to metabolic syndrome, and confirmed that *Nox4* functional missense variants were caused by SNPs in the CAST and PWK strains [88]. In future studies, we will dissect muscle transcriptome to identify candidate genes and genetic variants regulating muscle atrophy that are differentially expressed by Rg3, are associated with muscle atrophy-traits, and carry missense variants of PWK SNPs. Furthermore, we propose a strategy for phytochemicals to become candidates for the transition to human clinical studies using CC founder strains with diverse genetic backgrounds as bridges between the B6 strain and human trials. Candidate genes and genetic variants associated with phenotypic variation determined in phytochemical studies can be discovered through analysis methods, such as genome-wide association studies (GWAS) and quantitative trait loci (QTL) mapping. These approaches include genetic differences in the expression of enzymes that metabolize phytochemicals, metabolic pathways known to be affected by phytochemicals (i.e., mitochondrial energy metabolism), and nuclear transcription factors known to be influenced by phytochemicals (i.e., PGC1A and NRF1). With a better understanding of the association between the clinical response to phytochemicals and the genetic factors that determine these responses, targeted personal nutritional strategies that match the optimal phytochemical and genetic profiles can be developed to maximize the potential for clinical trial success.

Host genotype strongly influences metabolic disease-associated phenotypes, including muscle atrophy and bone loss. Utilizing the F2 cross, DO mice, and human studies, genetic variants associated with bone health have been reported through GWAS and QTL mapping [89–92]. The present study supports the notion that genetic factors influence muscle and bone health-related traits in response to Rg3, providing a broad range of responses within each strain. Among the traits measured in this study, muscle strength, muscle weight, endurance, and bone health, excluding spleen immune markers, showed a wide range of responses among the strains. Rg3 improves osteoporosis in the B6 strain [15–17], and in this study, among the six CC strains, Rg3 also increased BMD and bone area, although not significantly, in the DEX-induced B6 strain. However, in the AJ strain, these traits tended to decrease by Rg3 treatment. These results suggest that researchers need to move away from exclusively using B6 male mice simply for convenience and historical reasons. Interestingly, PWK responded best to Rg3. The utilization of wild-derived strains may allow the discovery of genetic factors that predict the potency of phytochemicals not found in more common laboratory strains.

Our findings also provide a model for phytochemical phenotyping studies to combine phenotyping data measured in genetically diverse mouse models with multi-omics data, including the genome, transcriptome, and gut microbiome. Combining the available GWAS data with the phenotype, gut microbiota, and transcriptome of a particular tissue allows for a better evaluation of SNPs associated with QTL mapping. However, limited studies have been conducted on the interaction between phytochemicals or other physiologically active dietary substances and genetic factors. In the future, it will be possible to study the interaction between phytochemical efficacy and genetic factors by mapping disease-associated phenotypes, microbial abundance, and gene expression to quantitative traits using CC and DO models and CC founder strains. These studies represent a broad and untapped new area of phytochemical study and will greatly advance our understanding of the mechanisms by which phytochemicals affect muscle atrophy and bone loss and the effects of individualized genetic determinants on the efficacy of these interventions.

While our study highlights that genetic diversity plays an important role in the response to muscle and bone health-related traits, there are several limitations in study design. First, the study tested only one natural compound, and many other compounds need to be investigated. In this study, Rg3 showed efficacy in mitigating muscle atrophy and bone loss, particularly in the PWK strain, but other phytochemicals may have beneficial effects on musculoskeletal traits in strains other than PWK. Secondly, the small number of mice per group is an obvious limitation to support our claims in terms of verifying the effectiveness of Rg3 in each strain. Unexpectedly, during the course of our experiments, the wild-derived strains CAST and WSB were excluded from the final analysis due to severe wounds, reducing our statistical power. However, a two-way ANOVA accounting for the strain and sample effects showed that Rg3 clearly ameliorated some muscle atrophy and bone loss-related phenotypes. The fact that these effects were more pronounced in NZO or PWK strains than in B6 strains suggests that the pharmacological effects of phytochemicals, including Rg3, should be validated in multiple strains before conducting clinical trials, given that most preclinical studies have been conducted in B6 strains. Thirdly, it is necessary to include the control (no DEX treatment) group in each strain to determine the extent to which muscle atrophy and bone loss were induced by DEX treatment relative to the control group. However, due to the high cost of mice imported from JAX, we were forced to exclude the control group from our study design for budgetary reasons. In our previous studies, we have successfully confirmed that candidate plant extracts or microbes successfully reduced grip strength and muscle weight by treating DEX (15 mg/kg, i.p. for 1 month) to C57BL/6J and 129S1/SvImJ strains (data not shown).

This study is the first to use nutrigenomics to investigate whether phytochemicals improve muscle atrophy and bone loss in strains with various genetic backgrounds. We also constructed a queryable interactive resource called Rg3-OsteoSarco. We demonstrated the ability of Rg3-OsteoSarco to easily identify the effects of Rg3 on each strain using the trait or trait–trait correlation query feature. We believe that the strain–phytochemical analysis tool included in Rg3-OsteoSarco will be a valuable resource for future researchers.

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CRediT authorship contribution statement

Bao Ngoc Nguyen: Methodology, Formal analysis, Writing - original draft. **Soyeon Hong:** Methodology, Formal analysis. **Sowoon Choi:** Methodology, Formal analysis. **Choong-Gu Lee:** Conceptualization, Methodology, Formal analysis, Writing - review & editing. **GyHye Yoo:** Conceptualization, Methodology, Formal analysis, Writing - review & editing. **Myungsuk Kim:** Supervision, Funding acquisition, Conceptualization, Methodology, Formal analysis, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no conflicts of interest in this work. Regarding the work submitted, we declare that we do not have any commercial or associative interests that would constitute a conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jgr.2023.12.004.

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