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Long-Lived Growth Hormone Receptor Knockout Mice Show a Delay in Age-Related Changes of Body Composition and Bone Characteristics

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There is conflicting information on the physiological role of growth hormone (GH) in the control of aging. This study reports dual-energy x-ray absorptiometry (DXA) measurements of body composition and bone characteristics in young, adult, and aged long-lived GH receptor knockout (GHR-KO) and normal mice to determine the effects of GH resistance during aging. Compared to controls, GHR-KO mice showed an increased percentage of body fat. GHR-KO mice have reduced total-body bone mineral density (BMD), bone mineral content, and bone area, but these parameters increased with age. In addition, GHR-KO mice have decreased femur length, femur BMD, and lower lumbar BMD compared to controls in all age groups. These parameters also continued to increase with age. Our results indicate that GH resistance alters body composition, bone growth, and bone maintenance during aging in GHR-KO mice.

GROWTH hormone (GH) plays an important role in post-natal growth and development. Humans and animals reach normal adult body size only in the presence of a functional GH axis. GH declines with age in rodents, primates, and humans (1–3). In addition, peripheral insulin-like growth factor I (IGF-I) also declines with age in rodents, primates, and humans (4–8). Because of the parallel decline in GH and bone mass with aging, a role for GH in maintaining a normal skeleton across life span is proposed. This idea is supported by the finding that patients developing GH deficiency in adulthood have decreased bone mineral density (BMD; 9). In addition, long-term GH deficiency untreated from childhood results in increased fractures (10). Evidence suggests that GH has a role throughout life in maintaining bone health.

A syndrome of GH resistance due to various mutations of the GH receptor has been described in humans (termed ''Laron Syndrome''), and this condition is characterized by severe growth reduction, delayed bone age, delayed puberty, obesity, hypoglycemia, osteopenia, increased serum levels of GH, and decreased circulatory levels of IGF-I (11). In 1997, Kopchick's group (12) developed GH receptor knockout (GHR-KO) mice (''Laron'' mice) that share many phenotypic similarities with humans deficient in GH signaling. Despite profound GH resistance, GHR-KO mice are healthy, can reproduce, and live significantly longer than their normal siblings (13–15).

The development of GHR-KO mice allows experiments to be designed to address the role of GH signaling in aging. GHR-KO mice exhibit extended average and maximal life span (13–15). In addition, they exhibit characteristics of delayed aging including a reduction in cognitive deficits in advanced age (16), a decrease in cataract development (17), and a decrease in neoplastic and non-neoplastic diseases (Y. Ikeno, A. Bartke, unpublished observations, 2005).

Little is known about the growth and maintenance of bone in the absence of an intact GH/IGF-I signaling system. It was previously shown that GHR-KO mice have reduced bone mineral content (BMC) at 24 weeks of age (18). In addition, 6-week-old GHR-KO mice had reduced cortical bone growth and trabecular bone turnover, and this reduction was reversible with IGF-I treatment (18). In addition to GH/IGF-I-mediated bone alterations with age, adiposity is thought to be altered by declining GH levels in the aging mammal (19–21). In the absence of an intact GH/IGF-I signaling axis, Berryman and colleagues (22) showed that GHR-KO mice have increased percent adiposity compared to normal controls at 6 months of age. To our knowledge, there are no studies regarding the effect of compromised GH/IGF-I signaling on bone characteristics and body composition during aging in the mouse. Our laboratory designed a cross-sectional study to examine the age-related alterations in body composition and bone density in GHR-KO mice compared to normal siblings at three different ages.

Methods

Animals

GHR-KO ''Laron dwarf'' mice were originally produced at Ohio University by Zhou and colleagues (12), who used homologous recombination targeting exon 4, which encodes

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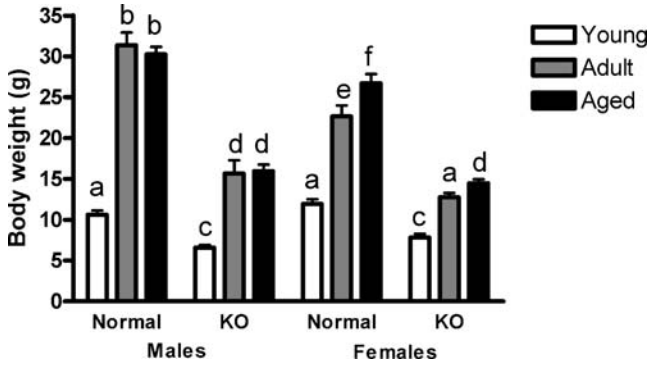

Figure 1. Body weight (grams) of male and female growth hormone receptor-knockout (GHR-KO) mice and their normal littermates across three age groups. Young animals were 6–7 weeks old, adult animals were 7–10 months old, and aged animals were 28–32 months old. The main effects of phenotype and sex on body weight from three-way analysis of variance were significant ($p < .0001$ for both), suggesting that GHR-KO phenotype and females weighed less overall. All data reported as means ± standard error of the mean. Groups that do not share a superscript are significantly different ($p < .05$). At least nine animals per group were analyzed.

a portion of the GH binding domain, to disrupt expression of this domain. This disruption leads to severe GH resistance. Mutations at the corresponding locus in the human have been associated with the clinical pathology known as ''Laron Syndrome'' or primary GH insensitivity (23). The homozygous (–/–) GHR-KO mice used in the present study were bred on a heterogeneous genetic background in a closed colony. Adult heterozygous (+/–) female mice were mated with heterozygous (+/–) or homozygous recessive (–/–) GHR-KO male mice.

Housing

GHR-KO (–/–) mice and their phenotypically normal (+/+ or +/–) littermates were maintained under controlled conditions of light (12-hour light/dark cycle) and temperature (22 ± 2°C) with ad libitum access to food (Lab Diet Formula 5001; Purina Mills, St. Louis, MO) and tap water. Animals were housed in shoebox style cages equipped with microisolator tops (five animals per cage). All animal procedures were approved by the laboratory animal care and use committee at Southern Illinois University School of Medicine. Sentinel animals were sent for bacterial and viral testing every 3 months.

Experimental Design

This study investigated the body composition and bone characteristics in young (6–7 weeks old), adult (7–10 months old), and aged (28–32 months old) GHR-KO mice and their normal controls. We used 137 mice in this experiment; 63 normal (32 male and 29 female) and 74 GHR-KO (37 male and 37 female).

Dual-Energy X-Ray Absorptiometry

Dual-energy x-ray absorptiometry (DXA) was used to analyze BMD, BMC, bone area, total body mass, body fat mass, percent fat, fat-free body mass, and percent lean mass using a PIXIMUS small animal densitometer and PIXIMUS software (PIXIMUS Lunar GE, Madison, WI). Both femur and lower lumbar (LL) bone were measured within a region of interest (ROI) to evaluate bones of known different ratios of trabecular/cortical content to further elucidate differences in bone characteristics between groups. In addition, femur length was obtained during measurements using the PIXIMUS software (1 pixel = 1.8 mm). All DXA measurements were obtained with the skull excluded, to increase accuracy as recommended by the manufacturer. All tails were included in measurements. Animals were lightly anesthetized using an intraperitoneal injection of ketamine HCl (100 mg/ml)/xylazine (20 mg/ml) mixture, at 0.1 ml per 10 grams of body weight. Animals were then placed on a sticky platform in ventral decubitus. Measurement of each animal was read in five replicates to assure reliable data acquisition. Quality controls included a phantom mouse as a calibration standard. Coefficient of variation (CV) for the phantom with 10 replicates was 0.21% for BMD. In addition, percent fat CV was 0.1%. Total body mass from whole-animal DXA and body weight determined using a conventional animal balance were in excellent agreement.

Statistics

Three-way analysis of variance was used to analyze the effects of phenotype, sex, and age, as well as the interaction of these variables to determine if mutation altered measured parameters at the different ages. Subsequent probable least squared differences (PLSD) were used to determine differences within groups. Differences across groups were determined using independent samples *t* test. All data are reported as means ± standard error of the means (SEM). Alpha (α) for each analysis was set at $p < .05$. All statistical evaluations were conducted using StatView 5.0.1 software (SAS Institute, Inc., Cary, NC).

Results

Body Weight and Body Composition

Figure 1 shows body weight for all groups studied. Analysis for the effects of age within each group separately (male normal, female normal, male GHR-KO, and female GHR-KO) revealed that adult and aged mice weighed more than their young counterparts ($p < .0001$) in all groups. In addition, both aged female normal and aged female GHR-KO mice showed an increase in body weight when compared to adults ($p < .0131$ and $p < .0227$, respectively). Surprisingly, body weight of aged males from normal and GHR-KO phenotypes did not differ from values measured in the corresponding adult cohorts ($p < .5013$ and $p < .8389$).

Body composition is a major determinant of animal health status. DXA measurements found that GHR-KO mice had an elevated total body percent fat compared to age- and sex-matched normal mice (Figure 2A). This finding was in contrast with total body percent lean results where there was no significant effect of phenotype ($p < .8525$).

Total body lean mass (% lean) in this study was calculated as fat-free mass minus bone tissue (see Figure 2B). This calculation differs from analyzing % lean with bone tissue included (data not shown), which is 100 – percent fat.

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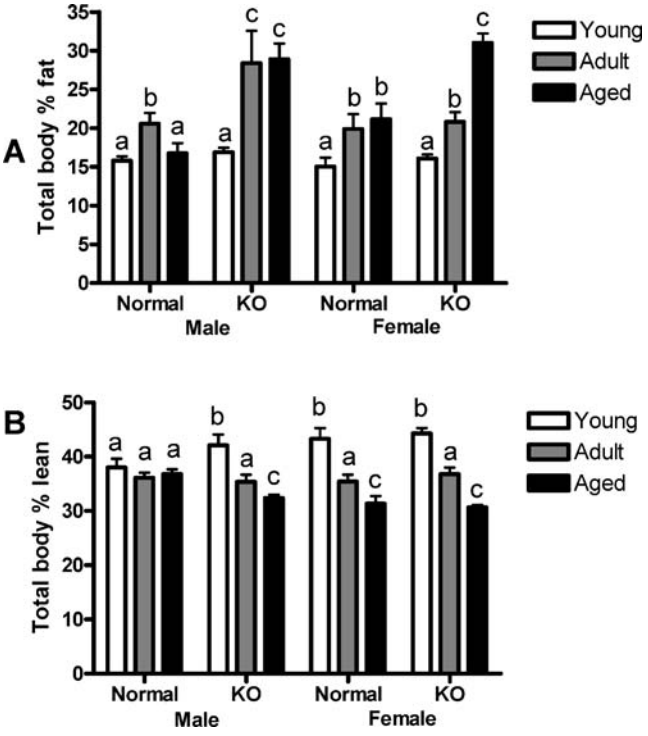

Figure 2. Total-body (**A**) percent fat and (**B**) percent lean tissue of male and female growth hormone receptor-knockout (GHR-KO) mice and their normal littermates. Lean tissue % is reported as fat-free mass minus bone tissue. Young animals were 6–7 weeks old, adult animals were 7–10 months old, and aged animals were 28–32 months old. Total body percent fat (% fat) was higher ($p < .0001$) in GHR-KO mice when compared to normal mice. Age was the only significant main effect ($p < .0001$) for total body % lean. All data reported as means ± standard error of the mean. Groups that do not share a superscript are significantly different ($p < .05$). At least nine animals per group were analyzed.

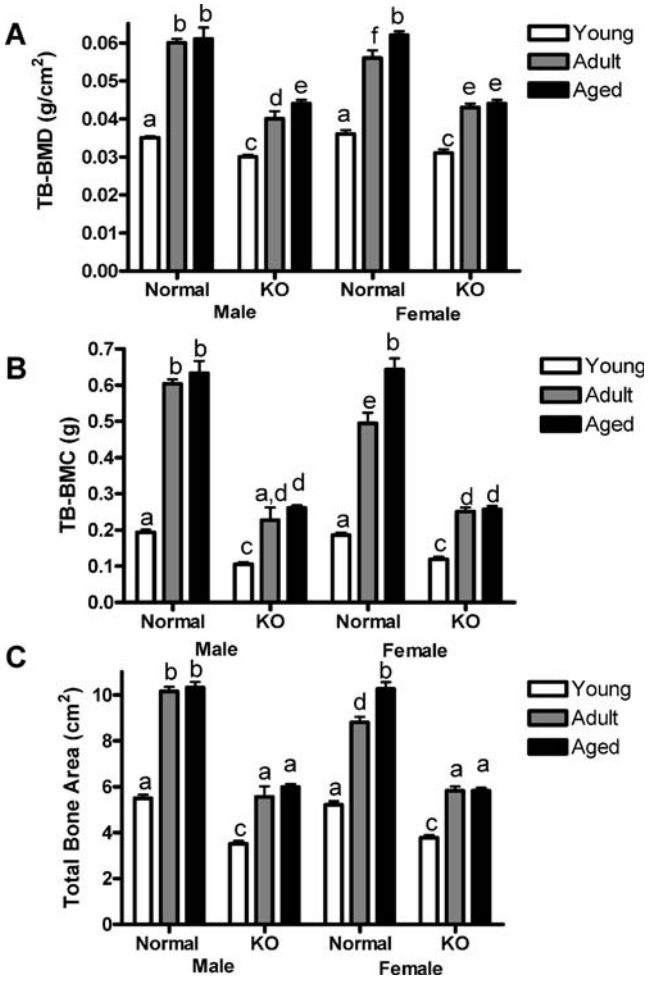

Figure 3. Total-body (TB) (**A**) bone mineral density (BMD) (g/cm^2), (**B**) bone mineral content (BMC) (g), and (**C**) bone area (cm^2), of male and female growth hormone receptor-knockout (GHR-KO) mice and their normal littermates. Young animals were 6–7 weeks old, adult animals were 7–10 months old, and aged animals were 28–32 months old. TB-BMD, TB-BMC, and TB-bone area was reduced in GHR-KO mice ($p < .0001$ for all). Age also resulted in an overall increase in TB-BMD, TB-BMC, and TB-bone area ($p < .0001$ in all cases). All data reported as means ± standard error of the mean. Groups that do not share a superscript are significantly different ($p < .05$). At least nine animals per group were analyzed.

Despite the results from three-way analysis of variance that suggested age as a major determinant of % lean tissue loss, when analyzed within phenotype male normal mice did not show a decline in % lean tissue with age.

Total Body Bone Characteristics

Aging is associated with increased fracture risk, which is associated with changes in bone properties reflected by altered bone density (10). Total-body BMD, BMC, and bone area were reduced in GHR-KO mice, regardless of age. Within the normal males and GHR-KO females, there was no significant difference in total-body BMD between adult and aged mice within groups ($p < .4462$ and $p < .3257$, respectively). In contrast, in GHR-KO males and normal females there was an increase in total-body BMD between adult and aged groups ($p < .0157$ and $p < .0024$, respectively).

BMC (grams) was approximately 3-fold higher in normal adult and aged mice, regardless of sex, when compared to the corresponding young normal animals, whereas adult and aged male and female GHR-KO mice showed only a 2-fold increase in total-body BMC compared to young age groups (see Figure 3B). In addition, there was a significant decrease in total-body BMC in GHR-KO when compared to normal mice, which is expected due to their decreased body size. There was an interaction effect of Phenotype * Sex * Age ($p < .0089$) in this analysis, suggesting that these three variables all contributed to altered total-body BMC. This parameter did not differ between adult and aged mice except for the normal females group, in which aged mice had higher total-body BMC than did adult mice (adult 0.495 ± 0.29 grams vs aged 0.643 ± .031 grams, $p < .003$).

Total-body bone area is a measure of total bone volume (cm^2) in the body. Normal aged female mice had higher bone area when compared to normal female adults ($p < .0010$), but there were no significant differences in bone area between adult and aged mice within any other group comparison (Figure 3C).

Femur and LL Bone Characteristics

To further delineate differences in bone characteristics between age and phenotype, we analyzed femur and LL

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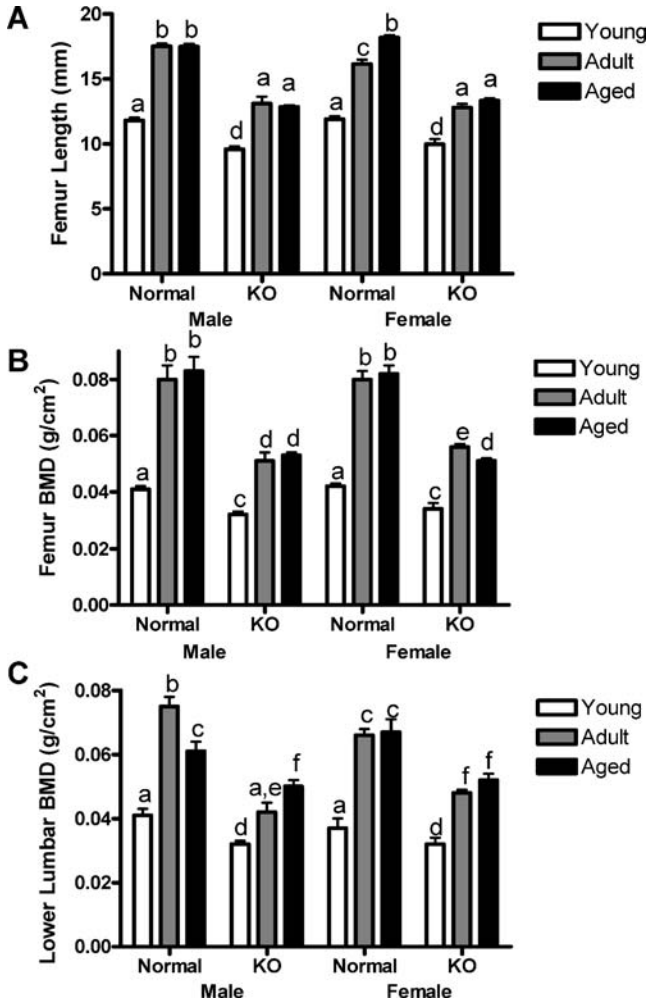

Figure 4. Femur length (mm) (**A**), femur bone mineral density (BMD) (g/cm^2) (**B**), and lower lumbar (LL) BMD (g/cm^2) (**C**) of male and female growth hormone receptor-knockout (GHR-KO) mice and their normal littermates. Young animals were 6–7 weeks old, adult animals were 7–10 months old, and aged animals were 28–32 months old. Femur length, femur BMD, and LL BMD were reduced at all ages in GHR-KO mice ($p < .0001$ in all cases). All data reported as means ± standard error of the mean. Groups that do not share a superscript are significantly different ($p < .05$). At least nine animals per group were analyzed.

bone alone. We chose these bones for their varying ratios of trabecular to cortical bone. As expected, there were reductions in all measured parameters tested in GHR-KO mice (Figure 4). Interestingly, there was an increase in femur length when adult or aged groups are compared to the corresponding young animals ($p < .0001$ for both comparisons). Further analysis within groups revealed a difference in adult versus aged mice in the normal female group only (16.2 ± 0.3 mm vs 18.2 ± 0.2 mm respectively, $p < .0001$). Surprisingly, femur BMD for female GHR-KO mice was actually reduced compared to adult mice (adult 0.056 ± 0.001 g/cm^2 vs aged 0.051 ± 0.001 g/cm^2, $p < .0197$) implying a relationship between sex and aging in GHR-KO mice.

Although LL BMD in normal versus GHR-KO mice followed similar trends, analysis within groups revealed a decrease in LL BMD (Figure 4C) in aged versus adult normal male mice only (adult 0.075 ± 0.003 g/cm^2 vs aged 0.061 ± 0.003 g/cm^2, $p < .0006$). In contrast, aged male GHR-KO mice had an increase in LL BMD compared to adults (adults 0.042 ± 0.002 vs aged 0.050 ± 0.002, $p < .0031$). In normal female and in female GHR-KO mice there were no differences in LL BMD between the adult and aged groups.

Discussion

In this study, we cross-sectionally measured age-related changes in body composition and bone characteristics in GHR-KO mice and their normal littermates. The GHR-KO mice are characterized as having primary GH resistance, reduced body growth, reduced plasma IGF-I, delayed onset of disease, and increased average and maximal life span (14). GH signaling deficiency, as expected, leads to a decrease in hepatic IGF-I expression and peripheral IGF-I levels. A role for reduced IGF-I signaling (or homologous signaling) has been implied in prolonging life in three taxonomically distant organisms: a nematode (*Caenorhabditis elegans*), a fly (*Drosophila melanogaster*), and a mouse (*Mus musculus*) (24). Body composition and bone characteristics are known to change with aging (25). Therefore, comparing age-related changes in body composition and bone characteristics in long-lived GHR-KO mice versus normal mice may produce some insights into the mechanisms by which GH signaling affects aging. The findings reported here compare GHR-KO mice to the age-matched and sex-matched normal littermates at three ages, young (6–7 weeks), adult (7–10 months), and aged (28–32 months).

Previous studies reported an increase in percent fat in adult GHR-KO mice compared to their age-matched normal littermates (22), but the effects of aging on this and other characteristics were not previously examined. Results obtained in the present study revealed that in young mice there were no major differences in body composition between the phenotypes other than the obvious size differences between the normal and GHR-KO mice. A novel finding in this article is that GHR-KO mice have a higher percent fat than normal controls have throughout adult life that continues into advanced age. These findings also differ from those in another long-lived mouse, the Ames dwarf (Prop1^{df}) mouse. The Ames dwarf mouse has a mutation that leads to GH deficiency and a profound reduction in peripheral IGF-I levels similar to findings in the GHR-KO mouse, but in addition it has thyroid-stimulating hormone (TSH) and prolactin deficiencies (26,27). Ames dwarf mice live 40%–60% longer than their normal littermates (28), and adult Ames dwarf mice have normal or reduced percent fat compared to controls (29). It is interesting that age-related alterations in body composition are different in these two long-lived mutant dwarf mice with reduced GH signaling. Both the Ames dwarf mice and GHR-KO mice have reduced plasma insulin and glucose levels (15,26,30). One could postulate that increased longevity in Ames dwarf mice could be attributed in part to reduced adiposity, which in turn leads to improved insulin signaling. However, the same concept would not apply to GHR-KO mice, as our results show an increase in adiposity with age. This is a conundrum because

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GHR-KO mice live longer than normal animals do, and obesity is believed to represent a major risk factor for age-related diseases (31,32). Berryman and colleagues (22) reported that GHR-KO mice have altered compartmental adiposity compared to age-matched normal mice. Namely, GHR-KO mice have an increased percentage of subcutaneous and retroperitoneal fat compartments but no relative difference in epididymal fat mass percentage. Further studies on the role of adipose tissue depots in mediating longevity and/or insulin sensitivity in these mutants are warranted.

Lean tissue changes were less dramatic than the changes in adiposity. Lean tissue percent decreased with age in all groups. Because bone is thought to follow muscle as a unit, it would be expected that bone density would also decrease with age (33). As lean tissue percent decreased in all groups (nonsignificant in normal males) from young to adult, it was not expected that total-body BMD would increase. However, as GHR-KO males and normal females aged, the total-body BMD increased even though percent lean tissue decreased. This finding indicates a separation within the muscle-bone unit normally seen during growth.

Femur length was decreased in the GHR-KO animals compared to sex-matched controls ($p < .0001$). The normal females had an increase in femur length from the adult time point to the aged time point. This was not seen in any of the other groups and indicates that sex dimorphism may be diminished in GHR-KO mice, at least with respect to this characteristic. However, an increase in estrogen during puberty has been shown to increase hepatic IGF-I concentrations in female GHR-KO mice (34). This does not appear to compensate for the deficiency of GH-stimulated IGF-I, because the female GHR-KO mice did not show femur growth from adult to aged groups as the normal females did.

An important cross-sectional finding in the present study is that GHR-KO mice have altered bone characteristics during aging which can be attributed to their GH resistance. Whereas, previous studies in young GHR-KO animals revealed decreased BMD and BMC, reduced longitudinal growth, reduced bone turnover, and overall disproportional skeletal growth (18,35,36), the present study shows that the reduction in BMC, BMD, bone area, and femur length persists throughout adult life and is also observed in aged individuals. This pattern of age-related changes may be a marker of delayed growth and subsequent delayed aging in GHR-KO mice. The LL BMD in male GHR-KO mice did not decrease with age as was seen in normal controls. This is most likely due to the decreased bone turnover rate previously reported (18).

There is a reduction in all aforementioned bone characteristics in GHR-KO mice compared to normal mice in each of the examined age groups. However, it should be emphasized that the absolute values measured in the aged GHR-KO mice resemble those of sex-matched young normal mice. Whether these findings suggest improved bone health and delayed aging, or if these reductions compared to normal mice are detrimental at all ages, remains to be determined. However, in view of the increased longevity of these mutants, aged GHR-KO mice having bone characteristics similar to those of young normal mice may be a positive attribute of this phenotype.

These data lead to many questions for future research. We did not address the turnover state of the bone tissue in this study. Bone turnover is thought to be a major contributor to bone loss associated with aging in humans. Likewise, decreased muscle mass is associated with decreased bone density of aging. Although % lean decreased, we did not measure force on the bone, and it may well have been sufficient to maintain bone density. Future studies measuring force generation across the bones as the mice age may help explain the bone-related changes.

Conclusion

Young, adult, and aged GHR-KO mice have increased whole-body adipose stores, reduced whole-body BMD, BMC, bone area, femur length, and femur BMD and LL BMD compared to age-matched and sex-matched controls. Though reduced in GHR-KO mice, these measured parameters increase with age, suggesting delayed aging and/or growth in this phenotype. In addition, bone characteristics of aged GHR-KO mice are similar to those of young normal mice in all groups tested. The present results indicate that GH resistance and subsequent deficiency of circulatory IGF-I alters bone growth and maintenance throughout aging in mice.

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