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IGF-1 REGULATES VERTEBRAL BONE AGING THROUGH SEX-SPECIFIC AND TIME-DEPENDENT MECHANISMS

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Abstract

Advanced aging is associated with increased risk of bone fracture, especially within the vertebrae, which exhibit significant reductions in trabecular bone structure. Aging is also associated with a reduction in circulating levels of insulin-like growth factor (IGF-1). Studies have suggested that the reduction in IGF-1 compromises healthspan, while others report that loss of IGF-1 is beneficial as it increases healthspan and lifespan. To date, the effect of decreases in circulating IGF-1 on vertebral bone aging has not been thoroughly investigated. Here, we delineate the consequences of a loss of circulating IGF-1 on vertebral bone aging in male and female *Igf1^{fl/f}* mice. IGF-1 was reduced at multiple specific time points during the mouse lifespan- early in postnatal development (crossing albumin-Cre mice with *Igf1^{fl/f}* mice), or early adulthood, and late adulthood using hepatic-specific viral vectors (AAV8-TBG-Cre). Vertebrae bone structure was analyzed at 27 months of age using microCT and quantitative bone histomorphometry. Consistent with previous studies, both male and female mice exhibited age-related reductions in vertebral bone structure. In male mice, reduction of circulating IGF-1 induced at any age did not diminish vertebral bone loss. Interestingly, early-life loss of IGF-1 in females resulted in a 67% increase in vertebral bone volume fraction, as well as increased connectivity density and increased trabecular number. The maintenance of bone structure in the early-life IGF-1-deficient females was associated with increased osteoblast surface and an increased ratio of osteoprotegerin/receptor-activator of NFkB-ligand levels in circulation. Within 3 months of a loss of IGF-1, there was a 2.2 fold increase in

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insulin receptor expression within the vertebral bones of our female mice, suggesting that local signaling may compensate for the loss of circulating IGF-1. Together, these data suggest the age-related loss of vertebral bone density in females can be reduced by modifying circulating IGF-1 levels early in life.

Keywords

Aging; IGF-1; Bone microCT; Bone histomorphometry; Osteoprotegerin

Introduction

Aging is associated with decreased bone structure and function, which dramatically increases the risk for osteoporosis and the development of bone fractures in elderly patients. Each year, nearly 2 million elderly Americans suffer fractures, with nearly 27% of these fractures occurring in the spine⁽¹⁾. Evidence accumulated over the past several years indicates that the age-related changes in bone health are the result of a shift from bone formation to bone resorption (as reviewed by^(2–4)). Osteoblast activity declines with age, while osteoclast activity increases. The changes in the balance of bone remodeling result in the loss of bone architecture, increasing the risk for osteoporosis and compression fractures. Several signaling hormones, including insulin-like growth factor-1 (IGF-1) and steroid hormones, have been shown to be important for bone health and remodeling through their direct actions on osteoblast activity or through other indirect mechanisms^(5–10).

Numerous clinical studies have reported a close association between circulating IGF-1 and bone mineral density^(11–18). During adolescence the rise in circulating IGF-1 that occurs in both rodents and humans is highly correlated with bone formation and skeletal growth. Similarly, an absence of IGF-1 (e.g. in homozygous IGF-1 knock-out mice) results in a significant retardation of bone and overall body growth as well as a variety of other complications that ultimately lead to premature death^(19–23). Knock-out of the receptor for IGF-1 in osteoblasts results in reduced bone volume and bone mineral density by adulthood^(24,25). Similarly, specific deletion of IGF-1 production in the liver results in a decrease in femoral bone length⁽²⁶⁾ and mineral density by adulthood⁽²⁷⁾. Femurs from circulating IGF-1-deficient mice also exhibit reduced bone strength and stiffness in adulthood⁽²⁸⁾. While these studies clearly highlight the importance of IGF-1 for proper bone development, the effects of a reduction in circulating IGF-1 on age-related changes in vertebral structure and function remains unknown.

Interestingly, higher levels of IGF-1 are not always associated with increased bone health. For example, early-life knockout of acid-labile subunit (ALS), an important stabilizing protein for IGF-1 in the circulation, has been shown to reduce IGF-1 levels by 60–75% but increase femur cortical thickness in aged male mice⁽²⁹⁾. These results suggest that an early and prolonged loss of IGF-1 may be beneficial for long bone aging. Contrary to these data, a recent study found that a decrease in circulating IGF-1 during adulthood leads to reduced cortical and trabecular bone thickness in the femur during aging⁽³⁰⁾. In this study, circulating IGF-1 was reduced at 12 months of age⁽³⁰⁾, and the results suggest that a loss of circulating

IGF-1 at mid-life is detrimental to long bone aging. Together, these disparate results suggest that the consequences of IGF-1 deficiency may be dependent on the stage of the lifespan when the deficiency is induced.

It is well-known that circulating levels of IGF-1 decrease with age^(31–34). The significance of this reduction remains controversial since some investigators provide evidence that replacement of IGF-1 to older animals and humans improves tissue function^(35–37) whereas others suggest that a reduction in IGF-1 may be beneficial since several species with reduced IGF-1 levels exhibit increased lifespan^(38–40). Recent findings indicating that femur structure is compromised when IGF-1 levels are reduced in adult male mice is consistent with the conclusion that loss of IGF-1 after mid-age is detrimental to bone health⁽³⁰⁾. Moreover, clinical studies suggest that reduced levels of IGF-1 in the circulation are associated with increased risk for osteoporosis^(16,17,41,42). Although there are only limited data available, the controversial effects of IGF-1 deficiency may be related to the age-of-onset with early IGF-1 deficiency having beneficial effects on tissue function whereas deficiency induced later in life has detrimental actions.

In the current study, our goal was to examine the effects of circulating IGF-1 on vertebral bone aging. Unlike previous studies, the current study included both male and female mice, since age-related changes in bone are often more pronounced in females. Due to the high incidence of age-related vertebral fractures in humans, we focused on vertebral bone, specifically lumbar vertebra (L4–5). To investigate the effects of age-of-onset of IGF-1 deficiency, circulating IGF-1 was reduced at multiple time points during the lifespan (early development, early adulthood, and late adulthood) and the reductions of IGF-1 were designed to be similar in magnitude to that occurring with age. Vertebral bone structure, along with a variety of circulating factors, was assessed at 27 months of age, which is an advanced age in this mouse strain. Our results provide clear evidence that the influence of circulating IGF-1 on vertebral bone aging is dependent on both sex and the age-of-onset of IGF-1 deficiency.

Materials and Methods

Animals

Male and female mice homozygous for a floxed exon 4 of the *Igf1* gene (*igf1^{f/f}*) were purchased from Jackson Laboratories (B6.129(FVB)-*Igf1*<tm1Dlr>/J) and backcrossed to C57Bl/6 for 6 generations in house. These mice have loxP sites flanking the entirety of exon 4, allowing for excision of this exon when exposed to Cre recombinase. The altered *Igf1* gene yields a protein that fails to bind the IGF receptor. Animals were housed (3–4 per cage) in Allentown XJ cages with Anderson's Enrich-o-cob bedding (Maumee, OH) in the specific pathogen free Rodent Barrier Facility at OUHSC on a 12h light / 12h dark cycle at 21°C. In this facility, all animals are free of helicobacter and parvovirus. Mice were given access to standard irradiated bacteria-free rodent chow (5053 Pico Lab, Purina Mills, Richmond, IN) and reverse osmosis water *ad libitum*. All procedures were approved by and followed the guidelines of the Institutional Animal Care and Use Committee of OUHSC.

The numbers of animals per group as well as the baseline information for each group (weight, age, IGF-1 levels) are outlined in Table 1. To target IGF-1 production early in development, *igf1^{ff}* mice were crossed with mice expressing Cre recombinase under an albumin-promoter, as previously described⁽⁴³⁾. The albumin gene is induced within the liver between post-natal day 10–15, thereby decreasing effective IGF-1 production early after birth (mice termed LID). To target IGF-1 production in adulthood, adeno-associated viruses (AAV8) were purchased from the University of Pennsylvania Viral Vector Core. While AAV8 is effective at transducing multiple tissues, the use of thyroxine binding globulin (TBG) promoter allows for the restriction of expression to hepatocytes, as described⁽⁴⁴⁾. At 5 or 15 months of age, *igf1^{ff}* mice were randomly assigned to treatment groups and administered approximately 1.3×10^{10} viral particles of AAV8-TBG-Cre or AAV8-TBG-eGFP via retro-orbital injection, as described⁽⁴⁴⁾. Briefly, mice were given an intraperitoneal injection of 20% ketamine/3% xylazine for anesthesia, per veterinary recommendation. After 5 minutes, 100µl of virus diluted in physiological saline was injected into the retro-orbital sinus. Mice were monitored in their home cage until they were fully-recovered from anesthesia. No mice exhibited adverse side effects of viral treatment. Wild-type C57/B16 male and female mice (3–4 months of age) were purchased from Jackson Laboratories (Bar Harbor, Maine) and utilized as young reference controls in the structural studies (n=3 per group). At the time of analysis, the order in which the mice were harvested was randomized using a block design.

Sera analysis

Whole blood was isolated from the submandibular vein and subsequently centrifuged at $2500 \times g$ for 20 minutes at 4°C to separate the serum. IGF-1, Osteoprotegerin, and RANKL concentrations within the sera were quantified using the Mouse IGF-1 Quantikine ELISA kit, Osteoprotegerin/TNRSF11B Quantikine ELISA kit, and TRANCE/RANKL/TNFSF11 Quantikine ELISA kit (all R&D Systems, Minneapolis, MN), respectively. GH levels in the sera were quantified using the Milliplex pituitary magnetic bead assay (EMD Millipore, Billerica, MA). IGF-1 was measured at multiple ages (2, 12, and 27 months of age), while OPG and RANKL were measured at 27 months of age. GH was measured at 7 and 27 months of age. All analytes were measured in replicate following the manufacturers' recommendations.

Bone MicroCT

As previously described^(45,46), the 5th lumbar vertebra was fixed in 4% formaldehyde and 70% ethanol and scanned using a high resolution Scanco vivaCT 40 µCT scanner (Scanco Medical, Bassersdorf, Switzerland) with a resolution of 10.5 µm. A total of 150 consecutive 10.5-µm-thick sections were analyzed. The segmentation values were set at 0.8/1/280. Three-dimensional reconstruction and structural parameters quantification were calculated using Scanco Medical software by a blinded researcher. Nomenclature for the microCT studies was derived from the previously established report⁽⁴⁷⁾.

Bone histomorphometry

Analysis of bone histomorphometry was performed by the Histomorphometry and Molecular Analysis Core in the Department of Pathology at the University of Alabama at

Birmingham. Following tissue isolation, the vertebrae samples were fixed in 4% formalin and subsequently trimmed in 70% ETOH. The samples were then processed for plastic embedding which included 4 changes of the infiltration solution (95% Methyl Methacrylate, MMA, and 5% Dibutyl phthalate, DBP) at three day intervals. After infiltration, samples were embedded in a solution of 95% MMA and 5% DBP with 0.25% perkodox as the initiator and exposed to UV light for polymerization. The fully-polymerized (plasticized) sample blocks were cut to obtain 5 μ m thick coronal sections. The sections were stained with Goldner's Trichrome for Histomorphometry (Bioquant Osteo 2014 software, Nashville, TN). The sections were quantified by two independent, blinded researchers. The length of the vertebrae was an average of the length along the outer edge of both sides of L5. Nomenclature for the histomorphometry studies was derived from the previously established report⁽⁴⁸⁾.

RNA Analysis

Gene expression of various targets (GH, GHR, IGF1, IGF2, IGFR, and InsR) was quantified in the QuantStudio 12k Flex (Applied Biosystems, Life Technologies, Waltham, MA) using Taqman Universal Master mix reagents (Life Technologies). Upon harvest, the bones were cleaned and immediately frozen in liquid nitrogen. The bones were then pulverized in liquid nitrogen using a Chemplex impact mortar and pestel, lysed in RNeasy lysis buffer, and sonicated. RNA was purified using the RNeasy kit and cDNA was prepared using Superscript III (Life Technologies). Taqman primers were purchased from Life Technologies. Table 2 includes a list of the primers used in this study. All data were normalized to a housekeeper gene, HPRT, and control GFP mice were set to 1 for fold comparison.

Statistics

Data were analyzed using SigmaPlot version 11 (Systat Software, San Jose, CA). Each animal was a unit of analysis. One-way ANOVA with post-hoc Dunnetts test was used to identify differences between the multiple treatment groups compared to aged control. Two-way ANOVA with a post-hoc Bonferroni multiple comparison was used to identify sex differences in combination with treatments. Unpaired t-tests were used to compare differences between two groups, as appropriate. Multivariate correlation assessment with a subsequent pairwise correlation analysis was performed in JMP statistical software (SAS, Cary, NC). Two outliers were identified by Mahalanobis distance in the correlation analysis of IGF-1, RANKL, OPG, and bone vertebrae- a 5 month Cre female and a 5 month GFP female (greater than 3 SD away from the group average). Based on this distribution, these mice were removed from subsequent analysis. For ease of comparison, the three aged control groups (wild-type, 5m GFP, and 15m GFP) were pooled and labeled control. No differences between these control groups were observed in any of the experiments. A p value less than 0.05 was considered statistically significant. Data are expressed as mean $\pm$ S.E.M.

Results

Circulating levels of IGF-1 were reduced at three distinct time points throughout the mouse lifespan- early development, early adulthood, and late adulthood (Figure 1A). To reduce

IGF-1 in early development, LID (Liver-IGF-1 Deficient) mice were generated by crossing *igf1^{fl/fl}* mice with mice expressing albumin-driven Cre⁽⁴³⁾. At post-natal day 10–15, albumin expression is activated in the liver, thereby lowering liver production of IGF-1⁽⁴³⁾. To reduce circulating IGF-1 in early and late adulthood, *igf1^{fl/fl}* mice were given retro-orbital injections of a liver-specific Cre, AAV8-TBG-Cre, or GFP. Circulating levels of IGF-1 were quantified at 2 months, 12 months, and at sacrifice (27 months) to verify knockdown (Table 1 and Figure 1B–C).

For ease of comparison, data from wild-type *igf1^{fl/fl}* mice that did not express the albumin-Cre gene were combined with wild-type *igf1^{fl/fl}* mice that received injections of AAV8-TBG-GFP. Thus, control refers to LID control, 5m GFP, and 15m GFP mice. No difference was observed between these three groups in any of the experimental tests. LID mice exhibited a significant reduction in IGF-1 early in life (82.8–85.5% compared to controls), which was maintained throughout the lifespan (Figure 1B–C). *Igf1^{fl/fl}* mice treated with AAV8-TBG-Cre at 5 or 15 months of age exhibited a significant reduction (81.4–89.3%) in circulating IGF-1 following their respective viral treatment (Figure 1B–C). The reduced IGF-1 associated with treatment was similar in male and female mice (Figure 1B–C). Aging itself has been shown to reduce IGF-1 levels, and control mice (wild-type, 5m GFP, and 15m GFP) exhibited a 32% reduction in circulating IGF-1 levels in both males and females by 27 months of age (Figure 1B–C).

Several previous studies have highlighted the unique feedback between IGF-1 and growth hormone (GH)- a loss of IGF-1 leads to compensatory increases in GH signaling⁽⁴⁹⁾. Therefore, GH levels in circulation were assessed in the aged, IGF-1 deficient mice. There were no significant differences in basal levels of GH in circulation of the 27 month old knock-down mice (male nor female), although there was a trend for increased GH in the IGF-1-deficient females (Table 1). These data were surprising as previous literature has shown GH levels increase when IGF-1 is reduced. Thus, we examined GH levels 3 months after knock-down (rather than at 27 months) and observed a two-fold rise in circulating GH in male mice, consistent with previous reports (Table 1). No changes in GH were observed in the female mice. Thus, a loss of circulating IGF-1 does increase GH levels in early adulthood, but these effects are lost by 27 months of age. It is important to note that the levels of GH were representative only of basal levels at the time of harvest rather than the pulsatile levels of GH that occur throughout the day.

Age and IGF-1 related changes in vertebral bone mass were assessed in 27 month old mice using microCT. Within males, aging (WT) was associated with a significant reduction in bone volume fraction (bone volume/tissue volume) compared to young mice (46.8%, Figure 2B), connectivity density (Figure 2C), and trabecular number (Figure 2E). Consistent with previous studies⁽⁵⁰⁾, aging was also associated with an increase in the structure model index (Figure 2D) and trabecular separation (Figure 2G). The increase in trabecular separation is likely explained by the age-related decrease in trabecular number (Figure 2E) and the absence of changes in trabecular thickness (Figure 2F).

While aging was associated with a variety of structural changes in the male vertebrae, the effect of reducing circulating IGF-1 was mixed. The loss of circulating IGF-1 (LID, 5m Cre,

and 15m Cre) resulted in significantly increased connectivity density compared to aged control mice (Figure 2C). Decreasing circulating IGF-1 in development (LID), early (5m Cre), or late adulthood (15m Cre) had no impact on bone as measured by bone volume fraction (Figure 2B) or the structure model index (Figure 2D). However, knock-out of circulating IGF-1 at any age lead to a significant reduction of trabecular bone thickness at 27 months, compared to the aged control mice (Figure 2E), suggesting that the age-related loss of bone is greater when circulating IGF-1 is reduced. Decreases in circulating IGF-1 at 15 months (15m Cre) of age lead to a significant increase in trabecular number as well as a corresponding decrease in trabecular separation at 27 months of age (Figure 2E and 2G). Early life knock-down (LID) and knock-down at 5 months (5m Cre) of age did not show this partial rescue effect.

Aging was also associated with decreased vertebral bone structure in female mice (Figure 3A). Bone volume fraction (bone volume / total volume) (Figure 3B), connectivity density (Figure 3C), and trabecular number (Figure 3E) were significantly decreased in aged females (WT) compared to young controls. Structure model index (Figure 3D) and trabecular separation (Figure 3G) were increased in aged female mice. No difference was observed in the trabecular thickness with age (Figure 3F). While males exhibited a 45% reduction in bone volume fraction with age, female mice exhibited a 54% reduction. This resulted in significantly lower bone volume fraction in aged females compared to aged males (0.09 vs 0.13, $p=0.003$).

The effects of circulating IGF-1 on vertebral bone structure in females were markedly different than that observed in male mice. Early-life reductions in IGF-1, both in the LID mice as well as the mice with IGF-1 knocked down at 5 months of age (5m Cre), led to increased bone volume fraction (bone volume / total volume) (Figure 3B), increased connectivity density (Figure 3C), and increased trabecular number (Figure 3E) compared to aged control mice. Decreasing IGF-1 at 5 months of age restored aged bone volume fraction and trabecular number to that observed in young reference females (t-test, $p=0.20$ and $p=0.68$, respectively). Consistent with these data, increased bone volume fraction and trabecular number in the early-life knock-down mice (5m Cre) was also associated with decreased structure model index (Figure 3D) and decreased trabecular separation (Figure 3G). Loss of circulating IGF-1 late in adulthood (15 m Cre) had no effect on the age-related changes in bone volume fraction, structural model index, or trabecular number (Figure 3B, 3D, 3E). Although there was no change in bone volume fraction, late onset IGF-1 deficiency (15m Cre) did lead to a significant reduction in trabecular thickness and as a consequence, an increase in trabecular separation (Figure 3F–G).

To further examine the effects of IGF-1 on vertebral bone structure, quantitative bone histomorphometry was employed. Because the reduction in IGF-1 beginning at 5 months of age had the most pronounced effect in females, we focused our histomorphometry studies on the aged *igf1^{fl/f}* mice treated with AAV8-TBG-GFP or AAV8-TBG-Cre at 5 months of age. Within females, the reduction in circulating IGF-1 levels resulted in a 10% reduction in longitudinal bone length, a 32% increase in bone volume fraction (bone volume / total volume), a 34% increase in trabecular number, as well as 38% reduction trabecular separation (Table 3), consistent with our microCT studies. Reduced IGF-1 in female mice

also led to decreased bone surface/ tissue volume and increased osteoblast surface (Table 3) with no significant effect on osteoblast or osteoclast number. In males, once again there were no differences between bone structure in the 5m GFP and 5m Cre mice (Table 3). It should be noted that female control mice exhibited significantly reduced trabecular number and increased trabecular separation compared to control male mice (Table 3), suggesting that the age-related trabecular bone loss was greater in the females, consistent with the microCT analysis. Decreased circulating IGF-1 in the female 5m Cre mice resulted in trabecular bone number and separation equal to the male counterparts (Table 3).

Because bone turnover and osteoblast and osteoclast activity are regulated by osteoprotegerin (OPG) and receptor-activator of NFkB ligand (RANKL) signaling, we next measured whether these factors are influenced by IGF-1. Female mice with decreased circulating IGF-1 beginning at 5 months of age resulted in a modest but non-significant decrease in RANKL levels (Figure 4A), a significant increase in OPG levels (Figure 4B), and a net significant increase in the ratio of OPG/RANKL at 27 months of age (Figure 4C). Consistent with our structural studies, reductions of IGF-1 at 5 months of age had no significant effect on the circulating levels of OPG, RANKL, or the ratio of OPG/RANKL in male mice (Figure 4D–F).

Correlation analysis of IGF-1, OPG, RANKL, and vertebral bone volume fraction across all aged mice within this study revealed that IGF-1 levels were negatively correlated with OPG and vertebral bone volume fraction in aged female mice (Table 4). These strong correlations are present despite the fact that the late-adulthood knockdown did not show the beneficial effects in bone structure as observed in the early-life knockdown mice. Once again, no significant correlations were observed in the male mice (Table 4), indicating that the effects of circulating IGF-1 on vertebral bone structure are sex-dependent as well as dependent on the age at which IGF-1 deficiency was induced.

Next, we addressed whether the increased bone fraction observed in the aged female knock-down mice was due to increased bone accumulation during late development or decreased bone loss during aging. For this, circulating IGF-1 was once again reduced in young (3–4 months of age) *igf1^{f/f}* mice using our viral constructs (AAV8-TBG-Cre or AAV8-TBG-GFP). Bone structure was analyzed using microCT after 3 months of knockdown, when the mice were at a stage of adulthood associated with peak bone mass. Interestingly, there was no difference in the structure of the female vertebrae with any of the parameters tested (Figure 5A–F), suggesting that the loss of IGF-1 at 3–4 months of age did not affect bone accumulation in adulthood. These bones were analyzed further to identify potential changes in the anabolic signaling pathways within the bones themselves following a loss of circulating IGF-1. Expression of local IGF-1 and IGF-2 was slightly, but not significantly increased in the vertebrae female knock-down mice (Figure 5G). There were no changes in the local mRNA expression of IGFR, GH, or GHR when circulating IGF-1 was reduced; however, female knock-down mice exhibited a 2.2 fold increase in insulin receptor (InsR) expression (Figure 5G). These data suggest that local insulin signaling may be increased in female mice when circulating IGF-1 levels are decreased. For comparison, expression of these anabolic signaling pathways was also quantified in the adult male knock-down mice. Unlike the females, there were no changes in the expression of InsR or any of the other

growth signaling pathways after three months knockdown of IGF-1 (Figure 5H). Together these data indicate that while peak bone mass is not altered when circulating IGF-1 is reduced in young females, there are potential compensatory changes in the growth signaling pathways at this early stage. These changes may account for reduced bone loss observed in the aged female knockdown mice.

Discussion

Our current study focused on understanding the effects of circulating IGF-1 deficiency vertebral on vertebral bone aging. Several previous studies have highlighted the importance of IGF-1 signaling in neonatal development of bone^(19–23); however, very few studies have examined the influence of IGF-1 on bone aging. No studies, to our knowledge, have examined the influence of IGF-1 on vertebral bone aging. Age-related bone loss is the leading cause of osteoporotic fractures in the elderly. Thus, understanding the mechanisms of bone aging is of critical importance. Our data demonstrate that within females, loss of circulating IGF-1 early in post-natal development (LID) and/or early in adulthood (5 months of age) leads to increased vertebral bone structure late in life (27 months). The protection of vertebral structure was not observed when IGF-1 was reduced later in life (15 months) in females or in males at any ages. Although the specific mechanisms for this effect remain to be determined, it is likely that compensatory endocrine or cell autonomous effects are induced in response to IGF-1 deficiency at least through 5 months of age that account for the maintenance of bone structure observed in females and that the ability to induce these compensatory events are lost with age.

The loss of IGF-1 in development is associated with a global reduction in overall animal size and significant reduction in vertebral and tibia bone length^(19,23,51). Consistent with this, we observed a 10% reduction in the overall length of the vertebrae in our aged IGF-1-deficient females. Considering that local IGF-1 production within the bone is important for bone development and mineralization (as reviewed by⁽⁵²⁾), it is possible that the loss of circulating IGF-1 only stunted longitudinal growth while local IGF-1 signaling was maintained or even increased, serving as an anabolic signal even in advanced age. This hypothesis would be supported by previous studies showing that the loss of circulating IGF-1 is associated with an increase in the local production of IGF-1 and the IGF-1 receptor in other cell types^(53,54). Within our study, the loss of circulating IGF-1 in female mice did not result in a significant increase in local production of IGF-1, IGF-2, or the IGF-1 receptor. However, each of these transcripts demonstrated a modest rise, perhaps shedding light on a potential general increase in anabolic signaling within the female vertebrae when circulating IGF-1 was knocked down.

Because the IGF-1 receptor has also been shown to have pro-anabolic effects following insulin activation⁽⁵⁵⁾, it is possible that the observed increases in bone structure were due to alterations in insulin signaling within the bone as well. A previous report indicated that a loss of IGF-1 signaling within osteoblasts resulted in increased insulin receptor signaling⁽⁵⁵⁾. Consistent with this, we observed a 2.2 fold increase in the expression of the insulin receptor within the vertebral bones of female mice after 3 months of IGF-1 knockdown. Because insulin receptor signaling has been shown to be an important anabolic regulator of bone

metabolism⁽⁵⁶⁾, it is likely that increased insulin receptor signaling contributes to the protection from age-related bone loss in our female IGF-1-deficient mice.

Increasing levels of OPG are also likely part of the mechanism contributing to the maintenance of bone volume fraction in females, since OPG is positively-correlated with bone mass and negatively-correlated with IGF-1. Considering the close relationship between OPG and estrogen levels^(9,57,58), it is not surprising that the alterations in OPG only occur in female mice. However, it is unknown whether estrogen levels are altered in our female IGF-1 deficient mice. We find this an unlikely outcome since IGF-1 levels have beneficial effects on ovarian function and loss of IGF-1 accelerates reproductive decline in female rats⁽⁵⁹⁾. It should be noted that although the female knock-downs were the only group to exhibit increased bone structure in advanced age, bone mass was only restored to the levels of their aged male counterparts. It is well-known that age-related bone loss is often more pronounced in females than in males⁽⁶⁰⁾ and this relationship was confirmed in the present study. Further research on the relationship between IGF-1 deficiency and age-related changes in estrogen levels will be required.

The production and release of IGF-1 from the liver into the circulation is regulated by GH; and the loss of IGF-1 results in a significant rise in circulating GH due to the loss of negative feedback at the hypothalamus and pituitary gland⁽⁴⁹⁾. Similar to IGF-1, GH has been shown to have anabolic effects on bone development and it has been suggested that the compensatory increases of GH when IGF-1 is reduced can prevent bone loss⁽⁶¹⁾. We initially hypothesized that early-life knock-down of circulating IGF-1 in females led to increased circulating GH levels, thereby protecting vertebral structure later in life. However, our analysis of basal levels of GH does not support this hypothesis. Following three months of knock-down, there were no changes in circulating GH in our female mice. At the time of harvest, when IGF-1 had been reduced for 22–25 months, GH levels in female IGF-1-deficient mice were slightly elevated; however this effect was not significant. Together, these data suggest that basal GH levels are not sufficiently elevated in our female IGF-1-deficient to account for the increase in bone mass. A more-thorough analysis of the pulsatility of GH following IGF-1 knockdown would be appropriate before any definitive conclusions on the role of GH are made. Our data also suggest that a local upregulation of GH signaling does not occur in the female IGF-1-deficient mice since three months following IGF-1 deficiency, there were no changes in the expression of GH or GHR in the vertebrae. It is possible that analysis of multiple timepoints would provide more insight into the molecular changes induced by loss of IGF-1; however, our current evidence does not suggest a role for GH signaling in protection of bone aging in our IGF-1-deficient female mice.

An additional protection mechanism that was not explored in this study is increased leptin signaling with early IGF-1 deficiency. IGF-1 has been shown to be a negative regulator of leptin, with nearly a two-fold increase occurring in circulating leptin in LID mice^(62,63). While the role of leptin on bone formation and remodeling has been controversial, a recent study has revealed that leptin signaling increases the production and function of osteoblasts⁽⁶⁴⁾. Thus, it is possible that within our early life female knockouts, enhanced leptin levels contribute to the protection from age-related bone loss. Once again, why this effect would be limited to females is not immediately obvious.

In this study, we have identified that early-life decreases in circulating IGF-1 within females are associated with increased vertebral bone structure in advanced aging. Because the rate and extent of age-related bone loss has been shown to be site-specific^(65–67), it may be interesting to examine whether our early-life IGF-1 deficient females exhibit similar protection within their long bones. As mentioned, previous studies have shown both protective and deleterious effects of IGF-1 deficiency on femoral aging^(29,30). These two studies used very different mouse models and the time points in which IGF-1 deficiency began in these two studies was markedly different, which may contribute to the divergence in results. The model presented here, in which we were able to target IGF-1 production at several points during the lifespan of both male and female mice, may tease apart the role of IGF-1 on long-bone aging as well. Nevertheless, the data presented here indicate that the effects of IGF-1 on vertebral bone aging appear to be temporally regulated and sex-specific. Along with the early data showing the complex effects of circulating IGF-1 on bone development, these data highlight the complex, multifaceted relationship between IGF-1 signaling and bone.

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Figure 1. Model of IGF-1 deficiency at various timepoints throughout lifespan

(A) Timeline of the onset of IGF-1 deficiency within mice in early post-natal development (LID, onset at 10–15 days, $n=7$), early adulthood (5m Cre, onset at 5 months, $n=6$), and late-adulthood (15m Cre, onset at 15 months, $n=4$). After the time of onset, IGF-1 levels remain low in circulation for the rest of life. Average levels of IGF-1 in circulation in male (B) or female (C) mice early in development (2 months), in adulthood (12 months), and at the end-of-life (27 months). The wild-type denotes data pooled from wild-type *igf1^{fl/f}*, 5m GFP, and 15m GFP mice. The asterisk indicates a significant difference compared to wild-type controls at that timepoint, while the pound sign indicates a significant difference in the wild-type mice compared to the young 2m time point, *# $p<0.05$, mean $\pm$ SEM.

Figure 2. Aging leads to decreased vertebral bone structure in males

(A) Representative images of the L4 vertebrae in male mice, as observed using μ CT. Average bone volume fraction (B), connectivity ($1/\text{mm}^3$) (C), structure model index (D), trabecular number ($1/\text{mm}$) (E), trabecular thickness (mm) (F), and trabecular separation (mm) (G) in the various treatment groups. The asterisk indicates a significant difference compared to the aged control mice, $*p < 0.05$, mean $\pm$ SEM.

Figure 3. Aging leads to decreased vertebral bone structure in females, which is restored by early-life IGF-1 deficiency

(A) Representative images of the L4 vertebrae in female mice, as observed using μ CT. Average bone volume fraction (B), connectivity density ($1/\text{mm}^3$) (C), structure model index (D), trabecular number ($1/\text{mm}$) (E), trabecular thickness (mm) (F), and trabecular separation (mm) (G) in the various treatment groups. The asterisk indicates a significant difference compared to the aged control mice, $*p < 0.05$, mean $\pm$ SEM.

Figure 4. Osteoprotegerin is elevated in aged, IGF-1 deficient female mice

Average OPG (A), RANKL (B) levels in the sera of the aged female mice. (C) The average ratio of OPG to RANKL in the aged female mice. Average OPG (D), RANKL (E) levels in the sera of the aged male mice. (F) The average ratio of OPG to RANKL in the aged male mice. The asterisk indicates a significant difference between groups, * $p < 0.05$, mean $\pm$ SEM, $n=4-8$.

Figure 5. Loss of circulating IGF-1 does not influence bone acquisition in female mice, but does lead to local upregulation of insulin receptor expression

Average bone volume fraction (A), connectivity density ($1/\text{mm}^3$) (B), structure model index (C), trabecular number ($1/\text{mm}$) (D), trabecular thickness (mm) (E), and trabecular separation (mm) (F) in the female *igf1^{fl/fl}* mice three months post injection of AAV8-TBG-Cre or AAV8-TBG-GFP. Average expression of several key anabolic signaling genes in the vertebrae of GFP and Cre-injected *igf1^{fl/fl}* mice, in both females (G) and males (H). The asterisk indicates a significant difference between groups, * $p < 0.05$, mean $\pm$ SEM, $n = 5-6$.

Table 1

Description of aged experimental animals

Sex	Cohort	Group	n	Age (Days)	Weight (g)	IGF-1 Levels in Circulation (ng/mL)	GH Levels in Circulation (ng/mL)
F	Aged (27mo)	Wild-type	7	821.29 ± 4.92	22.73 ± 1.43	286.72 ± 55.63	4.08 ± 2.5
F	Aged (27mo)	LIID	6	825.50 ± 1.64	18.59 ± 1.00*	47.37 ± 5.63*	6.79 ± 1.89 (p=0.13)
F	Aged (27mo)	5m GFP	7	821.57 ± 5.97	24.03 ± 1.67	314.93 ± 59.3	4.02 ± 1.77
F	Aged (27mo)	5m Cre	7	826.86 ± 0.38	19.18 ± 0.62*	40.60 ± 6.96*	6.50 ± 2.58 (p=0.2)
F	Aged (27mo)	15m GFP	5	822.60 ± 5.34	24.59 ± 1.23	268.98 ± 24.73	3.14 ± 1.77
F	Aged (27mo)	15m Cre	4	826.75 ± 2.63	17.47 ± 1.02*	47.20 ± 6.97*	5.83 ± 1.67 (p=0.27)
M	Aged (27mo)	Wild-type	7	821.29 ± 4.31	26.93 ± 1.71	318.50 ± 16.54	7.02 ± 3.2
M	Aged (27mo)	LIID	7	823.71 ± 2.93	23.44 ± 1.32*	46.26 ± 9.51*	10.05 ± 4.01 (p=0.56)
M	Aged (27mo)	5m GFP	7	825.29 ± 4.98	26.79 ± 0.98	292.91 ± 35.05	4.49 ± 3.9
M	Aged (27mo)	5m Cre	6	817.67 ± 5.92	23.22 ± 1.09*	54.43 ± 8.67*	7.98 ± 3.90 (p=0.53)
M	Aged (27mo)	15m GFP	5	823.00 ± 5.34	26.49 ± 1.05	312.81 ± 29.49	4.45 ± 4.07
M	Aged (27mo)	15m Cre	4	826.50 ± 1.73	24.53 ± 0.94*	56.00 ± 7.79*	4.24 ± 3.90 (p=0.87)
F	Young (7 mo)	Young GFP	6	195.83 ± 14.43	20.50 ± 1.25	353.77 ± 109.5	16.38 ± 5.25
F	Young (7 mo)	Young Cre	6	191.33 ± 18.59	20.04 ± 1.13	102.9 ± 35.00*	11.69 ± 6.89 (p=0.17)
M	Young (7 mo)	Young GFP	5	199.25 ± 6.5	----	406.93 ± 89.74	14.11 ± 4.05
M	Young (7 mo)	Young Cre	5	208.5 ± 0.57	----	128.52 ± 45.14*	36.16 ± 8.18*

Average age, weight, IGF-1 level and GH level at the time of harvest in each experimental group in both the aging study and the follow-up 3 month knock-down study.

The – indicates data not available.

The asterisk indicates a significant difference between the treatment group and its respective control group, *p<0.05, mean ± S.D.

Table 2

Description of primers used in this study.

Target	Assay #	Species	Target Region
InsR	Mm01211875_m1	mouse	Taqman-exon 1–2, amp 57
IGFR	Mm00802831_m1	mouse	Taqman-exon 17–18, amp 106
IGF1	Mm00439560_m1	mouse	Taqman-exon 2–3, amp 77
IGF2	Mm00439564_m1	mouse	Taqman-exon 2–3, amp 107
GHR	Mm00439096_m1	mouse	Taqman-exon 8–9, amp 116
GH	Mm00433590_g1	mouse	Taqman-exon 2–3, amp 56
HPRT	Mm01324427_m1	mouse	Taqman-exon 5–6, amp 108

Bone histomorphometry results of aged control mice and aged mice that received a viral vector to knockdown circulating IGF-1 production at 5 months of age.

Table 3

	Males			Females			GFP M vs F p value
	5m GFP	5m Cre	p value	5m GFP	5m Cre	p value	
Length mm	3.07 ± 0.08	2.96 ± 0.21	0.36	3.11 ± 0.15	2.82 ± 0.33	0.05	0.56
Bone Volume (BV) mm ²	0.37 ± 0.03	0.37 ± 0.02	0.99	0.388 ± 0.07	0.451 ± 0.04	0.25	0.84
Tissue Volume (TV) mm ²	2.93 ± 0.48	2.63 ± 0.74	0.54	3.316 ± 0.30	2.778 ± 0.15	0.08	0.39
BV/TV	12.83 ± 1.72	14.65 ± 3.23	0.37	11.271 ± 1.15	16.678 ± 2.42	0.03	0.36
Bone Surface (BS) mm	16.74 ± 2.45	15.79 ± 0.93	0.56	15.205 ± 2.32	18.464 ± 1.11	0.13	0.63
BS/BV (mm-1)	45.37 ± 5.86	42.90 ± 0.44	0.51	40.746 ± 2.42	41.766 ± 2.35	0.39	0.26
BS/TV (mm-1)	5.72 ± 0.12	6.26 ± 1.39	0.46	4.463 ± 0.34	6.744 ± 0.60	0.004	0.02
Trabecular Thickness (um)	44.78 ± 5.44	46.85 ± 0.49	0.55	50.334 ± 2.98	48.686 ± 3.03	0.36	0.23
Trabecular Number (mm-1)	2.86 ± 0.06	3.13 ± 0.69	0.46	2.232 ± 0.17	3.372 ± 0.30	0.004	0.02
Trabecular Separation (um)	304.89 ± 11.45	283.39 ± 73.78	0.58	415.439 ± 36.37	258.303 ± 31.05	0.005	0.04
Eroded Surface (ES) mm	0.65 ± 0.46	0.29 ± 0.07	0.24	0.815 ± 0.18	1.03 ± 0.21	0.23	0.59
Quiescent Surface (QS) mm	16.09 ± 2.01	15.51 ± 0.87	0.66	14.39 ± 2.16	17.46 ± 0.94	0.13	0.56
Osteoblast Surface (Obs) mm	5.70 ± 0.65	5.56 ± 2.21	0.93	5.071 ± 0.49	7.262 ± 0.28	0.003	0.52
Osteoclast Surface (OcS) mm	0.65 ± 0.6	0.29 ± 0.07	0.24	0.81 ± 0.18	1.028 ± 0.21	0.22	0.60
ES/BS	3.74 ± 1.95	1.82 ± 0.38	0.16	5.161 ± 0.76	5.453 ± 0.88	0.40	0.28
QS/BS	96.26 ± 1.95	98.18 ± 0.38	0.16	94.839 ± 0.76	94.734 ± 0.98	0.47	0.28
Obs/BS	33.76 ± 9.25	34.90 ± 12.64	0.89	35.517 ± 3.29	39.917 ± 2.72	0.17	0.76
OcS/BS	3.74 ± 1.95	1.82 ± 0.38	0.16	5.12 ± 0.75	5.256 ± 0.97	0.46	0.29
N.Ob/BS	27.51 ± 8.27	26.86 ± 10.23	0.93	31.429 ± 2.17	32.815 ± 1.96	0.33	0.38
N.Oc/BS	1.30 ± 0.80	0.43 ± 0.19	0.29	1.942 ± 0.41	1.636 ± 0.08	0.26	0.32

n= 5–6 in females, n=3–4 in males

Table 4

Correlation analysis of end-of-life IGF-1 concentration, OPG and RANKL levels, and vertebral bone mass, as measured by μ CT

Females			
	[OPG]	[RANKL]	BV/TV
[IGF-1]	-0.49 (0.001)	0.24 (0.58)	-0.42 (0.02)
[OPG]		-0.19 (0.03)	0.29 (0.02)
[RANKL]			-0.38 (0.02)
Males			
	[OPG]	[RANKL]	BV/TV
[IGF-1]	-0.03 (0.87)	0.32 (0.11)	-0.03 (0.87)
[OPG]		0.06 (0.73)	0.04 (0.82)
[RANKL]			-0.06 (0.98)

The large numbers indicate the r^2 value, while the numbers within the parentheses indicate the p values of the correlation comparison.