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# Extreme Obesity Due to Impaired Leptin Signaling in Mice Does Not Cause Knee Osteoarthritis

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***Objective.*** **To test the hypothesis that obesity resulting from deletion of the leptin gene or the leptin receptor gene results in increased knee osteoarthritis (OA), systemic inflammation, and altered subchondral bone morphology.**

***Methods.*** **Leptin-deficient (*ob*/*ob*) and leptin receptor–deficient (*db*/*db*) female mice compared with wild-type mice were studied, to document knee OA via histopathology. The levels of serum proinflammatory and antiinflammatory cytokines were measured using a multiplex bead immunoassay. Cortical and trabecular subchondral bone changes were documented by microfocal computed tomography, and body composition was quantified by dual x-ray absorptiometry.**

***Results.*** **Adiposity was increased by ~10-fold in *ob*/*ob* and *db*/*db* mice compared with controls, but it was not associated with an increased incidence of knee OA. Serum cytokine levels were unchanged in *ob*/*ob* and *db*/*db* mice relative to controls, except for the level of cytokine-induced neutrophil chemoattractant (keratinocyte chemoattractant; murine analog of interleukin-8), which was elevated. Leptin impairment was associated with reduced subchondral bone thickness and increased relative trabecular bone volume in the tibial epiphysis.**

***Conclusion.*** **Extreme obesity due to impaired leptin signaling induced alterations in subchondral bone morphology without increasing the incidence of knee OA. Systemic inflammatory cytokine levels remained largely unchanged in *ob*/*ob* and *db*/*db* mice. These findings suggest that body fat, in and of itself, may not be a risk factor for joint degeneration, because adiposity in the absence of leptin signaling is insufficient to induce systemic inflammation and knee OA in female C57BL/6J mice. These results imply a pleiotropic role of leptin in the development of OA by regulating both the skeletal and immune systems.**

Obesity is a primary risk factor for osteoarthritis (OA); however, there is currently no comprehensive explanation for why obesity increases the risk of OA at sites throughout the body. Most hypotheses focus on mechanical factors, because the risk of developing knee OA increases with increasing body weight (1). However, a systemic factor may be involved, because a portion of epidemiologic studies show that OA at non–load-bearing joints, such as the hand, is also associated with obesity, albeit to a lesser extent than at the knee (2). Body fat itself may be the systemic mediator of an OA outcome. Increasing evidence in support of this hypothesis comes from recent studies showing that adipose tissue is not simply an inert energy storage depot, but rather it is an active endocrine organ that secretes numerous cytokines and cytokine-like molecules termed adipokines. Excessive centrally located adipose tissue is implicated as a major source of proinflammatory adipokines due to the infiltration of activated macrophages associated with adipocyte necrosis (3). Consequently, obesity is now considered a mild, chronic inflammatory disease. In addition to mediating inflammation, adipokines regulate systemic metabolic, skeletal, and reproductive processes (4). One adipokine in particular, leptin, is known to influence all of these varied processes (5).

Leptin is a 16-kd polypeptide hormone encoded by the obese (*ob*) gene (5). Leptin is primarily secreted by adipocytes, and it regulates adipose tissue mass and body weight by functioning as an afferent signal in a

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negative-feedback loop involving the hypothalamus (5). Mutations in either the *ob* gene or the gene encoding the leptin receptor (i.e., the diabetes, or *db*, gene) result in severe obesity. Impaired leptin signaling also results in increased axial bone mass via a central mechanism involving a hypothalamic relay and neural output (6). The effect of leptin on bone is heterogeneous within the body; leptin-knockout mice have increased lumbar vertebral bone mass and decreased femoral bone mass (7). Leptin-mediated bone remodeling may be relevant to the development of OA, by inference from the known association of subchondral bone thickening and remodeling with progression of cartilage degeneration (8).

Perhaps the most intriguing link between leptin and the pathogenesis of OA is the role of leptin in mediating inflammatory processes. For example, Otero and colleagues showed that costimulation of chondrocytes with leptin and interleukin-1 (IL-1) or interferon-$\gamma$ increased the expression of inducible nitric oxide synthase and produced a synergistic increase in nitric oxide production (9,10). Nitric oxide mediates the effects of IL-1 on joint degradation by down-regulating matrix synthesis and up-regulating matrix metalloproteinase (MMP) activity (11). Further evidence that leptin mediates catabolic processes comes from a study by Iliopoulos and colleagues, who showed that silencing leptin gene expression in severely arthritic cartilage reduced MMP-13 gene expression by half (12). However, leptin has also been shown to exert anabolic effects in articular cartilage by stimulating the production of 2 growth factors, transforming growth factor $\beta$ and insulin-like growth factor (13). These findings are significant, because both anabolic and catabolic activities of chondrocytes are up-regulated with the development of OA.

Given the well-established relationship between body mass index (BMI) and the risk of developing OA, particularly of the knee (1,2), the strong correlation between the serum leptin concentration and body fat is consistent with a pro-degenerative role of leptin. Furthermore, with leptin concentrations in synovial fluid exceeding those in serum (14,15), local sources of leptin production in the joint or factors affecting leptin clearance may be of particular importance in understanding how leptin affects joint health. Synovial fluid leptin concentrations were significantly correlated with BMI in persons with severe OA (13), and leptin gene expression was also significantly correlated with BMI in severely arthritic cartilage (15). Moreover, women were shown to have higher concentrations of free leptin in the joint when compared with men of similar age and BMI (14), which is consistent with the observation that OA is more likely to develop in women as they age.

These studies support a role of leptin as a metabolic link between obesity and altered articular cartilage metabolism. Although there is strong evidence that obesity induced by a high-fat diet in C57BL mice accelerates OA (16–19), it is not known whether impaired leptin signaling in vivo alters the development of spontaneous age-dependent degenerative changes in the joints of mice. Morbid obesity develops in both leptin-deficient (*ob*/*ob*) and leptin receptor–deficient (*db*/*db*) mice, thereby providing models in which to examine the relationships between leptin signaling, obesity, and OA. Although obesity is a strong risk factor for OA, the proinflammatory effects of leptin and its effects on bone mass suggest that impaired leptin signaling may mitigate joint degeneration. To address these questions, we characterized degenerative changes in the knee joint, quantified epiphyseal bone structure, and measured serum cytokine levels in female *ob*/*ob*, *db*/*db*, and C57BL/6 wild-type (WT) control mice.

## MATERIALS AND METHODS

**Animals.** Female WT (C57BL/6J; n = 15), *ob*/*ob* (B6.V-*Lep*$^{ob}$/J; n = 6), ob/+ (B6.V-*Lep*$^{ob}$/+; n = 5), and *db*/*db* (B6.Cg-*m*+/+ *Lepr*$^{db}$/J; n = 5) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and were housed in the Duke University Vivarium. Mice were housed in groups of 2–4 per cage and were kept on a 12-hour light/12-hour dark cycle, with unlimited access to food and water for the duration of the study. At 10–12 months of age, mice were anesthetized intraperitoneally with pentobarbital (60 mg/kg) and scanned for body composition analysis. At this time, blood was collected for serum cytokine analysis, resulting in exsanguination. Death was confirmed via thoracotomy. Following death, the limbs were dissected and immediately frozen in phosphate buffered saline. All procedures were performed in accordance with a protocol approved by the Duke University Institutional Animal Care and Use Committee.

**Body composition.** Lean body mass and body fat content of the mice were measured using a dual x-ray absorptiometry system (PIXImus2; Faxitron X-ray, Wheeling, IL). The percent body fat was measured as the body fat content, excluding the head, divided by the total body mass.

**Histologic analysis.** Knee joints were thawed and fixed in 10% buffered formalin for microfocal computed tomography (micro-CT) evaluation. Following micro-CT evaluation, intact knee joints were decalcified, dehydrated, and embedded in paraffin. Serial sagittal 6-$\mu$m sections were collected throughout the medial and lateral condyles. Sections were stained with hematoxylin, fast green, and Safranin O, and sections in the tibiofemoral cartilage–cartilage contact region from the medial and lateral condyles were scored for degenerative changes, using a modification (20) of the Mankin scoring system (21). Briefly, this scoring system included

**Table 1.** Body mass and adiposity in the different groups of mice*

| | Leptin intact, WT | Leptin impaired | | |
|---|---|---|---|---|
| | | *ob*/+ | *ob*/*ob* | *db*/*db* |
| Body mass, gm | 23.8 ± 0.8 | 32.8 ± 0.9 | 83.5 ± 2.4 | 73.8 ± 3.0† |
| Body fat, gm | 4.0 ± 0.4 | 6.9 ± 0.4 | 43.4 ± 1.5 | 39.7 ± 2.7† |
| Body fat, % | 16.3 ± 1.0 | 21.0 ± 0.8 | 52.0 ± 0.6 | 53.6 ± 1.8 |
| Peritoneal fat, gm | 0.5 ± 0.1 | 1.9 ± 0.2 | 3.2 ± 0.4 | 3.7 ± 0.4 |

* Values are the mean ± SEM. For all comparisons of intact versus impaired, $P = 0.01$. For all comparisons of *ob*/+ versus wild-type (WT), $P < 0.05$. All $P$ values were determined using nested analysis of variance.
† $P < 0.05$ versus *ob*/*ob*.

changes in articular cartilage structure (score of 0–11), Safranin O staining (score of 0–8), tidemark duplication (score of 0–3), fibrocartilage (score of 0–2), chondrocyte clones in uncalcified cartilage (score of 0–2), hypertrophic chondrocytes (score of 0–2), and relative subchondral bone thickness (score of 0–2), for a maximum score of 30 per location. Scores were determined by averaging values assigned under blinded conditions by 3–5 experienced graders for each of 4 locations in the joint: lateral femur, lateral tibia, medial femur, and medial tibia.

**Micro-CT skeletal analysis** . To quantify the effects of impaired leptin signaling on knee joint skeletal morphology and material properties, joints from each mouse were scanned using a micro-CT system (microCT 40 and vivaCT; Scanco Medical, Basserdorf, Switzerland). A global thresholding procedure was used to segment calcified tissue from soft tissue. Linear attenuation values for the calcified tissue were scaled to bone density values (mg of hydroxyapatite/$cm^3$) using a hydroxyapatite calibration phantom. Morphometric parameters of fully calcified cortical and trabecular bone in the tibial epiphysis were determined using a direct 3-dimensional (3-D) approach in the region distal to the subchondral bone and proximal to the growth plate.

The following parameters were determined for the tibial epiphysis: cortical bone volume ($BV_{cort}$, $cm^3$), total volume (TV, $cm^3$), relative cortical bone volume ($BV_{cort}/TV$), cortical bone density (mg hydroxyapatite/$cm^3$), trabecular bone volume ($BV_{trab}$, $cm^3$), total trabecular volume ($TV_{trab}$ [$TV - BV_{cort}$]), relative trabecular bone volume ($BV_{trab}/TV_{trab}$), and trabecular bone density (mg hydroxyapatite/$cm^3$). Subchondral thickness was determined by creating 2-D sagittal section images from the 3-D rendering of each joint. Images were obtained at the midpoint of the medial and lateral calcified meniscus, which approximates the tibiofemoral cartilage–cartilage contact region. Images were imported into ImageJ (NIH Image, National Institutes of Health, Bethesda, MD; online at: http://rsbweb.nih.gov/ij/), and subchondral thickness was measured by averaging 3 measurements in the central third of the subchondral region along the anterior–posterior direction. Thickness was measured at each of 4 locations in the joint: lateral femur, lateral tibia, medial femur, and medial tibia.

**Serum cytokine, chemokine, and biomarker analysis.** Blood was collected from anesthetized mice and dispensed into BD Vacutainer SST serum tubes (no. VT6514; VWR International, Morrisville, NC). After ~30 minutes, the blood samples were centrifuged for 10 minutes at 3,500 revolutions per minute, and the serum was stored in aliquots at −80°C until analyzed. Levels of serum leptin were quantified by a sandwich enzyme-linked immunosorbent assay (ELISA) (no. EZML-82K; Linco, Bedford, MA) specific for the detection of mouse leptin. The minimum detectable concentration of leptin is reported as 0.05 ng/ml. The intraassay and interassay coefficients of variation were 3% and 2.7%, respectively.

The following cytokines and chemokines were measured in the serum, using a multiplex bead immunoassay (BioSource, Fleurus, Belgium) specific to mice, with the Luminex 100 instrument (Luminex, Austin, TX): IL-1$\alpha$, IL-1$\beta$, IL-2, IL-4, IL-6, IL-10, IL-12, IL-17, cytokine-induced neutrophil chemoattractant (keratinocyte chemoattractant [KC]; mouse analog of IL-8), and tumor necrosis factor $\alpha$ (TNF$\alpha$). All samples were analyzed as recommended by the manufacturer. For samples that were below the level of detection, a number that was one-half the value of the lowest level of quantification was assigned for the purpose of performing statistical analyses. Levels of hyaluronic acid (HA) in sera were quantified with a commercially available ELISA (Corgenix, Westminster, CO) that utilizes HA binding protein as a capture molecule. The HA molecule is universal rather than species specific; thus, the assay accurately detects HA levels in nonhuman samples. The minimum detectable concentration of HA is reported as 10 ng/ml. The intraassay and interassay coefficients of variation were 4.2% and 6.2%, respectively.

**Statistical analysis.** A nested 2-level analysis of variance (ANOVA) was used to determine the statistical significance of differences in the mean values attributable to either impaired leptin signaling (level 1: pooled *ob*/*ob* and *db*/*db* versus WT) or strain (level 2: *ob*/*ob* versus *db*/*db*). Cytokine data were logarithm transformed to correct for non-normal distributions. Although a few other parameters were not normally distributed, reanalyses with the nonparametric Wilcoxon test did not alter the original ANOVA results. Statistical tests were performed using JMP 7.0 software (SAS Institute, Cary, NC). All data are reported as the mean ± SEM.

## RESULTS

**Body composition and joint histologic changes in leptin-impaired mice.** Disruption of leptin signaling resulted in a >3-fold increase in body mass and an

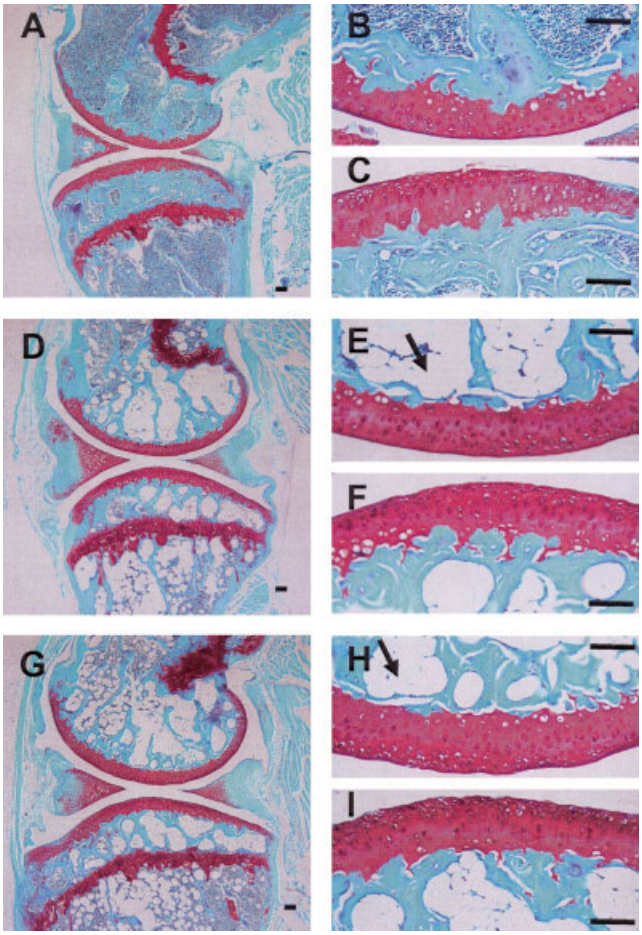


**Figure 1.** Safranin O–, fast green–, and hematoxylin-stained sagittal sections of knee joints from 44-week-old female wild-type mice (**A–C**), *db*/*db* mice (**D–F**), and *ob*/*ob* mice (**G–I**). The higher-magnification views are images of the central regions of the mediofemoral condyle (**B, E,** and **H**) and the tibial plateau (**C, F,** and **I**). **Arrows** show examples of lipid deposits in the intertrabecular region of the epiphysis. Bars = 100 $\mu$m.

~10-fold increase in body fat (Table 1). Heterozygote (*ob*/+) mice showed a smaller (30–40%) increase in body mass and fat (Table 1). Increased adiposity was also observed in the bone marrow cavities of the distal femoral epiphysis and the proximal tibial epiphysis and metaphysis of leptin-impaired mice (Figure 1).

Impaired leptin signaling did not, however, increase articular cartilage degeneration. There was no difference either in site-specific OA scores (Figure 2) or in the OA scores from each location summed together to obtain a total average degenerative joint score per strain (for WT, mean ± SEM 18.5 ± 1.9; for *ob*/*ob,* 19.5 ± 1.9; for *db*/*db,* 14.9 ± 2.7 [$P = 0.67$, impaired versus intact]). Analysis of the individual components of the modified Mankin scoring system showed that impaired leptin signaling did not affect cartilage structural changes (for WT, mean ± SEM 4.88 ± 0.80; for *ob*/*ob*, 7.53 ± 1.02; for *db*/*db*, 5.12 ± 0.78 [$P = 0.18$, impaired versus intact]) (maximum value of 44 for summed site scores) or the loss of Safranin O staining intensity (for WT, mean ± SEM 8.68 ± 1.58; for *ob*/*ob,* 8.33 ± 1.04; for *db*/*db,* 5.56 ± 1.64 [$P = 0.83$, impaired versus intact]) (maximum value of 32 for summed site scores). However, 2 of the components of the modified Mankin scoring system for OA were different between WT and leptin-impaired mice: leptin-impaired mice had fewer hypertrophic chondrocytes ($P < 0.04$) and reduced subchondral bone thickness ($P < 0.01$). Site-specific OA scores, total average scores, structural changes, and loss of Safranin O staining intensity did not differ between *ob*/+ and WT mice (data not shown). However, *ob*/+ mice showed significantly fewer cartilage structural changes compared with *ob*/*ob* mice (mean ± SEM 3.73 ± 0.54 versus 7.53 ± 1.02; $P = 0.013$).

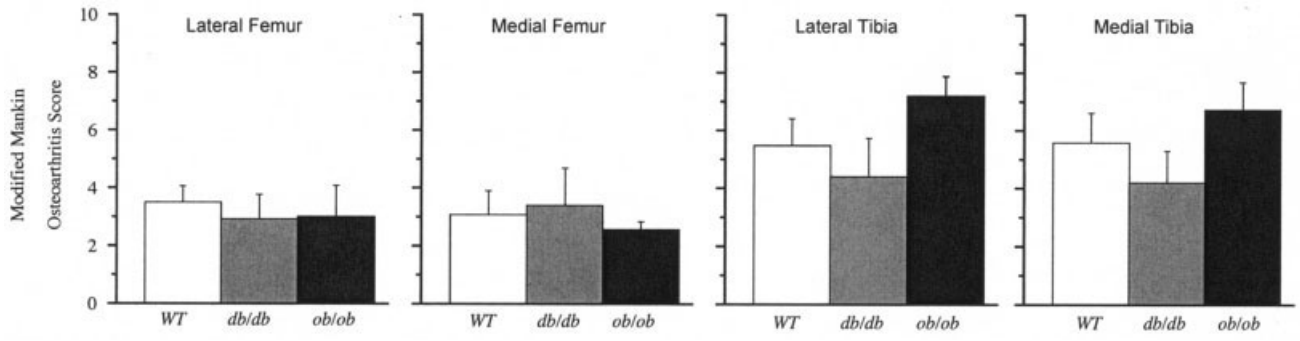


**Figure 2.** Modified Mankin scores for histologic changes in articular cartilage in knee joints from leptin-intact (wild-type [WT]) and leptin-impaired (*db*/*db* and *ob*/*ob*) mice. Bars show the mean and SEM.

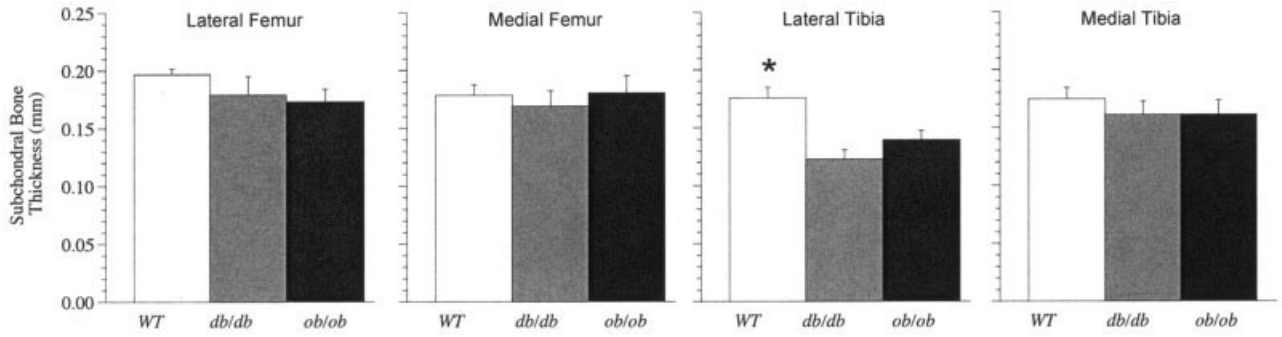


**Figure 3.** Subchondral bone thickness in knee joints from leptin-intact (wild-type [WT]) and leptin-impaired (*db*/*db* and *ob*/*ob*) mice. Bars show the mean and SEM. $* = P < 0.01$ versus impaired.

**Effect of impaired leptin signaling on skeletal joint morphology and bone mineral density.** To determine how impaired leptin signaling affected skeletal joint structure, the subchondral bone region and tibial epiphysis were examined in detail. Subchondral bone thickness was generally reduced in *ob*/*ob* and *db*/*db* mice, with the greatest reductions occurring in the lateral compartment of the knee (for the lateral femur, $P = 0.09$; for the lateral tibia, $P < 0.01$) (Figure 3). However, the overall relative cortical bone volume ($BV_{cort}/TV$) in the tibial epiphysis was not significantly altered in *ob*/*ob*, *db*/*db* (Table 2), or *ob*/+ mice. In contrast, the relative trabecular bone volume ($BV_{trab}/TV_{trab}$) in the tibial epiphysis was ~25% greater in *ob*/*ob*, *db*/*db* (Table 2), and ob/+ mice (mean ± SEM 0.54 ± 0.02). This finding was associated with a trend toward increased $BV_{trab}$ values in leptin-impaired mice. Impaired leptin signaling was not associated with changes in subchondral cortical bone density or trabecular bone density in the tibial epiphysis (Table 2).

**Serum cytokines and markers of cartilage degeneration.** Consistent with their genotypes, WT, *ob*/*ob*, and *db*/*db* mice had significantly different serum leptin levels, namely, low levels in *ob*/*ob* mice and high levels in *db*/*db* mice compared with WT controls (Table 3). Of the 7 proinflammatory cytokines measured in the serum, only the level of KC was significantly different ($P < 0.05$) between control and leptin-impaired mice, being ~2.8-fold greater in leptin-impaired mice (Table 3). There was also a trend for *ob*/*ob* and *db*/*db* mice to have lower levels of IL-2 compared with WT mice. Impaired leptin signaling had no significant effect on basal circulating levels of IL-1$\alpha$, IL-1$\beta$, IL-6, IL-17, or TNF$\alpha$. However, IL-1$\alpha$ levels were significantly greater in *db*/*db* mice compared with *ob*/*ob* mice.

Of the 3 antiinflammatory cytokines measured in

**Table 2.** Tibial epiphysis bone volume and density in the different groups of mice*

| | Leptin intact, WT (n = 11) | Leptin impaired | |
|---|---|---|---|
| | | *ob*/*ob* (n = 6) | *db*/*db* (n = 5) |
| Cortical bone | | | |
| Bone volume, $cm^3$ | 0.20 ± 0.02 | 0.17 ± 0.03 | 0.20 ± 0.01 |
| Total epiphyseal volume, $cm^3$ | 0.62 ± 0.06 | 0.58 ± 0.11 | 0.60 ± 0.03 |
| Relative bone volume | 0.33 ± 0.02 | 0.29 ± 0.01 | 0.33 ± 0.01 |
| Bone density, mg hydroxyapatite/$cm^3$ | 1,118 ± 9 | 1,074 ± 13 | 1,102 ± 17 |
| Trabecular bone | | | |
| Bone volume, $cm^3$ | 0.17 ± 0.02 | 0.23 ± 0.04 | 0.22 ± 0.01 |
| Total epiphyseal volume, $cm^3$ | 0.42 ± 0.04 | 0.42 ± 0.08 | 0.41 ± 0.02 |
| Relative bone volume | 0.43 ± 0.02† | 0.57 ± 0.03 | 0.53 ± 0.03 |
| Bone density, mg hydroxyapatite/$cm^3$ | 1,039 ± 12 | 1,020 ± 3 | 1,036 ± 9 |

* Values are the mean ± SEM. Except where indicated otherwise, differences between the leptin-intact group and the leptin-impaired group were not significant.
† $P < 0.001$ versus impaired.

**Table 3.** Serum concentrations of leptin, cytokines, and hyaluronic acid in the different groups of mice*

| | | Leptin impaired | |
|---|---|---|---|
| | Leptin intact, WT (n = 13) | *ob*/*ob* (n = 6) | *db*/*db* (n = 5) |
| Leptin, pg/ml | 6.06 ± 1.31† | 1.02 ± 0.01 | 169.3 ± 32.9‡ |
| Proinflammatory cytokines, pg/ml | | | |
| IL-1$\alpha$ | 979.4 ± 205.5 | 323.5 ± 61.7 | 1,153.6 ± 329.1‡ |
| IL-1$\beta$ | 80.9 ± 49.8 | 82.7 ± 48.2 | 76.0 ± 67.9 |
| IL-2 | 151.8 ± 77.0 | 6.0 ± 0.0 | 6.0 ± 0.0 |
| IL-6 | 79.0 ± 37.4 | 6.1 ± 0.0 | 16.7 ± 10.6 |
| IL-17 | 52.6 ± 35.7 | 9.3 ± 0.0 | 95.5 ± 53.1 |
| KC | 243.5 ± 63.7§ | 391.8 ± 45.7 | 524.9 ± 57.2 |
| TNF$\alpha$ | 16.5 ± 1.7 | 14.8 ± 0.0 | 70.8 ± 56.0 |
| Antiinflammatory cytokines, pg/ml | | | |
| IL-4 | 9.4 ± 0.0 | 9.4 ± 0.0 | 21.3 ± 9.4‡ |
| IL-10 | 91.5 ± 70.6 | 20.9 ± 0.0 | 20.9 ± 0.0 |
| IL-12 | 278.0 ± 50.5 | 214.2 ± 54.6 | 229.9 ± 74.2 |
| Hyaluronic acid, ng/ml | 431.6 ± 43.8 | 327.5 ± 90.4 | 487.8 ± 283.1 |

* Values are the mean ± SEM. Values below the lowest level of quantification (LLQ) were given a value of 0.5 × LLQ. Statistical analyses were conducted on $\log_{10}$ values to correct for non-normal distributions. All $P$ values were determined using nested analysis of variance. Except where indicated otherwise, differences between the leptin-intact group and the leptin-impaired group were not significant. WT = wild-type; KC = keratinocyte chemoattractant (mouse analog of interleukin-8 [IL-8]); TNF$\alpha$ = tumor necrosis factor $\alpha$.
† $P < 0.01$ versus leptin impaired.
‡ $P < 0.05$ versus *ob*/*ob*.
§ $P < 0.05$ versus leptin impaired.

the serum, there was a trend for the level of IL-4 to be greater in leptin-impaired mice compared with controls (Table 3). This result was solely attributable to elevated levels of IL-4 in *db*/*db* mice compared with *ob*/*ob* mice ($P < 0.05$). There were no other leptin-signaling or strain-related differences in antiinflammatory cytokine levels.

In addition to measuring markers of inflammation, circulating levels of HA were measured as a biomarker of cartilage degradation. Serum levels of circulating HA did not differ between leptin-impaired mice and their controls or between the leptin-impaired *ob*/*ob* and *db*/*db* strains (Table 3). Furthermore, the values obtained in the present study were comparable with values in C57BL/6 mice (20) and were lower than those for the intervention group (546.8 ng/ml) in that study, i.e., a model of trauma-induced knee OA (20). There were, however, moderate correlations between HA levels and degenerative changes occurring in the knee joint. These correlations were greatest for degenerative changes in the lateral compartment. Lateral femur and tibia OA score correlations with HA were $r_s = 0.49$ and $r_s = 0.45$ ($P = 0.04$ and $P = 0.06$, respectively; correlations were calculated using the nonparametric Spearman's correlation coefficient test).

## DISCUSSION

Obesity is a significant risk factor for OA in both weight-bearing and non–weight-bearing joints (1,2,22). Here, we report that older mice lacking leptin signaling due to nonfunctional circulating leptin (*ob*/*ob*) or nonfunctional leptin receptors (*db*/*db*) develop extreme obesity phenotypes without an increased incidence of knee OA. These findings suggest that body fat alone may not be a risk factor for joint degeneration, but rather, that other local and systemic factors are responsible for the relationship between obesity and OA. In weight-bearing joints, such as the knee, much progress has been made in identifying relationships between mechanical factors and the onset and progression of OA (23,24). However, mechanical factors seem less likely to explain the increased risk of OA in non–weight-bearing joints, implicating the involvement of a systemic factor. Recent studies suggest that leptin may be that obesity-linked systemic factor because of the proinflammatory effects of leptin in cartilage and its elevated levels in OA joints (9,10,12–15).

It is surprising that the incidence of knee OA was unchanged in *ob*/*ob* and *db*/*db* mice, given their dramatic obese phenotype—they weighed more than 3 times as

much and had ~10-fold more body fat compared with age-matched controls (Table 1). Studies using diet-induced models of obesity show that feeding C57BL mice a high-fat diet increases the incidence of OA in the knee despite a much less severe obese phenotype (16–19). Thus, given the severity of obesity in leptin-impaired mice, it seems likely that both local (e.g., mechanical) and systemic (e.g., metabolic or inflammatory) factors would promote the development of knee OA.

The fact that the incidence of knee OA was not increased is consistent with the interpretation that leptin influences the pathogenesis of knee OA directly rather than being correlated with obesity. This interpretation is also supported by 2 additional observations. First, hyperphagia-induced obesity, which is caused by administration of aurothioglucose and results in reduces hypothalamic leptin signaling (25), did not increase the incidence of OA (26). Second, we observed that heterozygosity for the *ob* gene, which resulted in reduced plasma leptin concentrations, increased body mass, and increased fat mass compared with WT mice (27) (Table 1), did not increase the incidence of knee OA. Intriguingly, cartilage structural changes in *ob*/+ mice were reduced relative to those in *ob*/*ob* mice, suggesting that the potential chondroprotective effects of reduced leptin signaling can be modified by other factors.

Discerning a direct role for leptin in the pathogenesis of OA is difficult, because nonspecific disruption of leptin signaling produces phenotypes that may be primary, secondary, or tertiary to the interruption in brain and peripheral tissue signaling pathways that regulate energy homeostasis (28). Nevertheless, the resultant phenotype provides a model for interpreting the relationship between obesity-related pathologies and OA, with and without intact leptin signaling. Furthermore, the severity of weight gain in leptin-impaired mice may provide a model of altered joint loading associated with morbid obesity.

Body mass and the body mass index are associated with changes in the magnitude and orientation of joint loading and the subsequent development of knee OA in humans (23,24,29). We observed medial–lateral differences in subchondral bone thinning between leptin-impaired and leptin-intact mice, specifically decreased subchondral bone thickness in the lateral, but not medial, compartments in leptin-impaired mice. This pattern of an increased ratio of medial-to-lateral subchondral bone thickness in leptin-impaired mice (Figure 3) is consistent with observations in overweight humans (30) and may be related to altered joint-loading patterns.

As in obese humans (31,32), leptin-impaired mice load their joints less frequently and likely generate joint stresses that are much less proportional to their body weight compared with controls. The *ob*/*ob* mice have significantly reduced levels of spontaneous activity (33), and muscular forces, which contribute significantly to joint stresses, are likely reduced in *ob*/*ob* mice due to reduced skeletal muscle contractile dynamics and mass (34). Furthermore, their large abdominal fat deposits may unweight the limbs by providing significant body weight support (Griffin TM, et al: unpublished observations). It is not clear to what extent these changes in musculoskeletal loading affect the pathogenesis of OA in leptin-impaired mice. A reduction in loading may seem to be protective; however, increased physical activity in mice and humans does not necessarily increase the incidence of OA (35,36).

We investigated the circulating serum levels of proinflammatory and antiinflammatory cytokines to determine how systemic inflammation status was affected by excessive adiposity in *ob*/*ob* and *db*/*db* mice. Adipose tissue is a potent source of proinflammatory and antiinflammatory cytokine production (4), which may promote catabolic processes that link obesity with OA (14,37). Leptin-impaired mice, however, were not in a generalized state of inflammation, as indicated by comparable serum levels of proinflammatory cytokines in leptin-impaired and leptin-intact mice (Table 3). Only the level of KC, a CXC chemokine and human IL-8 analog that functions as an inflammatory chemoattractant (38), was elevated in leptin-impaired mice. Furthermore, the level of IL-2 was reduced in leptin-impaired mice, similar to what is observed in obese humans (39). Additionally, impaired leptin signaling did not dramatically affect serum levels of antiinflammatory cytokines except IL-4, the level of which was elevated in *db*/*db* mice.

The lack of association between obesity and inflammation in *ob*/*ob* mice, while perhaps surprising given their extreme adiposity, is consonant with research showing that leptin deficiency modulates immune function (40). Leptin deficiency increases sensitivity to innate (i.e., monocyte/macrophage-activating) immune responses (41), whereas it decreases sensitivity to acquired (i.e., T cell–mediated) immune responses (42). These effects of leptin on the immune response have been demonstrated by 2 recent studies targeting innate versus acquired immune-mediated arthritis in *ob*/*ob* mice (43,44). The overall similarity in serum cytokine levels and knee OA scores in leptin-impaired and control mice is consistent with a hypothesized relationship between systemic inflammation and obesity-associated OA. Fu-

ture studies are needed to determine the relationship between systemic and local (i.e., intraarticular) inflammation.

Leptin may also mediate the development of OA via central and peripheral mechanisms that regulate bone mass (6,45). In *ob*/*ob* mice, leptin deficiency results in a mosaic bone mass phenotype, with bone mass being increased in the axial skeleton and decreased in the appendicular skeleton (7). Although the relationship linking altered bone remodeling to OA is complex, being dependent on both the OA model and the degree of disease progression (46), OA is typically associated with increased subchondral bone mass (i.e., sclerosis) as well as osteophyte growth in the joint periphery. We observed that leptin deficiency produced a mosaic bone phenotype in the joint with respect to subchondral cortical versus trabecular bone (Table 2).

Leptin-impaired mice showed regional subchondral bone thinning without changes in the overall relative cortical bone volume or density in the proximal tibial epiphysis. In contrast, the trabecular bone volume was increased in the proximal tibial epiphysis. Thus, unlike the femoral neck region in which cortical bone thickness and trabecular bone volume are reduced (7), the proximal tibial epiphysis exhibits similarities with the lumbar vertebrae of *ob*/*ob* mice, manifesting increased trabecular bone volume and decreased subchondral cortical bone thickness relative to WT controls (7). The extent to which this phenotype is influenced by altered joint loading patterns is unknown. However, our observation that the relative trabecular bone volume is also increased in the tibial epiphysis of *ob*/+ mice, similar to that observed in vertebral bodies of *ob*/+ mice (6), supports the notion of a direct role of the involvement of leptin signaling in mediating trabecular bone morphology in the knee. Thus, leptin signaling appears to regulate both cortical and trabecular bone mass in ways that may be relevant to OA pathogenesis.

Leptin may further regulate tissue mineralization by targeting chondrocytes. In the growth plate, leptin is localized in prehypertrophic chondrocytes, and the leptin receptor is localized in hypertrophic chondrocytes (45). Leptin deficiency, as observed in *ob*/*ob* mice, increases hypertrophic chondrocyte apoptosis and impairs endochondral ossification (45). In the current study, we observed that *ob*/*ob* and *db*/*db* mice had significantly fewer hypertrophic chondrocytes in the calcified cartilage of the tibia. Dumond et al (13) previously showed that rat articular chondrocytes express leptin receptors, and that injections of leptin into the knee joint increase the expression of transforming growth factor $\beta$1, insulin-like growth factor 1, and leptin messenger RNA. These localized proanabolic effects of leptin, when considered in conjunction with the proposed inflammatory and procatabolic effects of leptin, are consistent with an overall increase in anabolic and catabolic activities of chondrocytes in OA.

An additional way in which leptin may mediate the etiology of OA is via its actions on the reproductive system. Administration of leptin protects against infertility in *ob*/*ob* mice (47). Articular chondrocytes express functional estrogen receptors (48), and the concurrence of a spike in the onset of OA with menopause has implicated an OA-protective effect of estrogen in women. Many animal models of ovariectomy and estrogen treatment also show a protective effect of estrogen on OA pathogenesis (49). Although little is known about the interaction between leptin and estrogen in OA, both ovariectomy and menopause precede increases in adiposity (50,51), indicating that OA associated with these high-to-low estrogen transitions is also associated with increasing levels of leptin. Interestingly, *ob*/*ob* and *db*/*db* mice have low estrogen levels, impaired leptin signaling, and an unaltered incidence of knee OA.

Several additional factors should be considered when interpreting our findings. First, given the significant variation in susceptibility to obesity and OA among different mouse strains, additional studies with leptin-impaired mice created on different background strains are needed to generalize the findings from this study. Furthermore, a high-fat diet pair-feeding experimental design for comparing WT and leptin-deficient mice would provide additional weight and dietary controls for evaluating the protective effects of impaired leptin signaling on the development of OA.

In conclusion, the incidence of knee OA is not increased in extremely obese leptin-impaired mice. This finding is consistent with recent studies that implicate leptin as a proinflammatory and procatabolic mediator of OA associated with obesity (9,10,12–15). Leptin, however, has many pleiotropic effects on the body, including significant roles in the musculoskeletal, immune, and reproductive systems. Our findings indicate that impaired leptin signaling significantly alters subchondral bone morphology without altering knee OA, suggesting that obesity, other obesity-dependent factors, or the absence of leptin signaling independently moderates subchondral bone morphology. Furthermore, adiposity alone, in the absence of leptin signaling, is insufficient to induce systemic inflammation. Additional insight into the potential chondroprotective effects of disrupting leptin signaling may be obtained by examining

leptin-impaired mice in models of acute OA, such as instability or injury models.

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## AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Guilak had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

**Study conception and design.** Griffin, Huebner, Kraus, Guilak.
**Acquisition of data.** Griffin, Huebner, Kraus.
**Analysis and interpretation of data.** Griffin, Huebner, Kraus, Guilak.

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