ORIGINAL ARTICLE

Lengthening of mouse hindlimbs with joint loading

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Abstract For devising clinical approaches to treating limb length discrepancies, strategies that will generate differential longitudinal growth need to be improved. This report addresses the following question: does knee loading increase bone length of the loaded hindlimb? Knee loading has been shown to induce anabolic responses on the periosteal and endosteal surfaces, but its effects on longitudinal bone growth have not yet been examined. In the present studies, loads were applied to the left hindlimb (5-min bouts at 0.5 N) of C57/BL/6 mice (21 mice, ∼8 weeks old). Compared to the contralateral and age-matched control groups, knee loading increased the length of the femur by 2.3 and 3.5%, together with the tibia by 2.3 and 3.7% (all $P < 0.001$), respectively. In accordance with the length measurements, knee loading elevated BMD and BMC in both the femur and the tibia. Histological analysis of the proximal tibia revealed that the loaded growth plate elevated its height by 19.5% ($P < 0.001$) and the cross-sectional area by 30.7% ($P < 0.05$). Particularly in the hypertrophic zone, knee loading increased the number of chondrocytes ($P < 0.01$) as well as their cellular height ($P < 0.001$) along the length of the tibia. Taken together, this study demonstrates for the first time the potential effectiveness of knee loading in adjusting limb length discrepancy.

Keywords Femur · Tibia · Knee loading · Bone length

Introduction

Limb length discrepancy is caused by many reasons including bone injury, bone infection, inflammatory and noninflammatory diseases, and neurological disorders [1, 2]. Such limb abnormalities vary as to age of onset. Some are congenital (from birth), some developmental, from a childhood disease, or some, in the elderly, the result of posttraumatic incidents and osteoporosis [3, 4]. Approximately 25% of a typical population exhibits a length difference of 1 cm or more in their lower limbs [5]. Although the effects of such a discrepancy differ depending on the individual's age and stature, treatments are usually recommended for differences greater than 2 cm [6]. Except for use of shoe lifts, most of the currently available treatments are both surgical and invasive [7, 8]. In general, bones have the capability to remodel when the surgical treatment is not excessive. There are, however, risks associated with surgery, including premature consolidation, delayed union, and infection. This study was designed to evaluate the usage of mechanical loading (knee loading) as a potential noninvasive treatment.

Knee loading is a recently developed loading modality that applies lateral loads to the knee [9–11]. Using a relatively small magnitude of force (0.5 N to mice) compared to other loading modalities, anabolic responses in the femur

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and the tibia have been reported with rodent models [12]. Those previous studies, however, focused on the periosteal and endosteal surfaces. To our knowledge, no studies have examined effects on longitudinal growth using joint loading.

In the present study we addressed the question as to whether knee loading would alter the length of a hindlimb. It was hypothesized that knee loading would lengthen both the femur and the tibia with an increase in the height of the growth plate. Using C57/BL/6 mice (∼8 weeks of age), we determined bone length, bone weight, bone mineral density (BMD), and bone mineral content (BMC) of treated and contralateral hindlimbs. To investigate the potential mechanism of bone lengthening, we conducted histological analysis focusing on the proliferative and hypertrophic zones of the growth plate. Because treatment of limb length discrepancy is clinically relevant in young populations, the age of mice in the current study was chosen to be ∼8 weeks.

Materials and methods

Animals

Experimental procedures were approved by the Indiana University Animal Care and Use Committee and were in compliance with the Guiding Principles in the Care and Use of Animals endorsed by the American Physiological Society. Twenty-one C57/BL/6 female mice (∼8 weeks of age; Harlan Sprague-Dawley, Indianapolis, IN, USA) were used. Four to five mice were housed per cage; they were fed with mice chow and water ad libitum. The animals were allowed to acclimate for 2 weeks before experimentation.

Knee loading

Twenty-one mice were divided into two groups: the knee loading group ($n = 15$) and the age-matched control group ($n = 6$). Body weight was measured before initial loading and at death. During loading, the mouse was placed in an anesthetic induction chamber for sedation and mask-anesthetized using 1.5% isoflurane. A custom-made piezo-electric mechanical loader was employed to apply lateral loads for 3 min/day for 10 days (5 days/week) to the left knee in the lateromedial direction (Fig. 1). Loads with a peak-to-peak force of 0.5 N were given at 5 Hz [9–11]. The right hindlimb was used as a sham-loading (contra-lateral) control, in which the same treatment regimen to the left hindlimb was given without any dynamic loading. Similarly, the same procedure was applied to the age-matched control group without application of lateral loads.
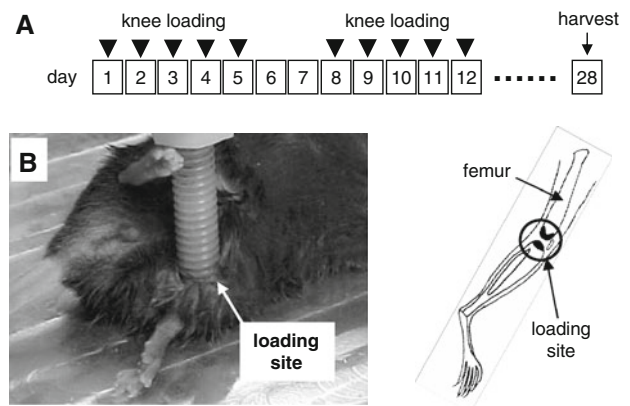
Fig. 1 Experimental setup. a Timeline for knee loading (ten loading bouts). b Loading site for knee loading

After loading, the mouse was allowed normal cage activity and then killed 2 weeks after the last loading.

Measurements of bone length, bone weight, BMD, and BMC

After death, femora and tibiae were harvested. These bone samples were cleaned of soft tissue and fixed in 10% neutral buffered formalin. The lengths of the femur and the tibia were determined using a digital caliper at 10-μm resolution (Mitsutoyo). Femoral length was defined as the maximum distance from the distolateral condyle to the most medial and proximal position on the femoral head. Tibial length was defined from the most proximal position of the tibial plateau to the most distal position of the medial malleolus. Bone wet weight was determined immediately after length measurements with an electronic balance [13]. Using the procedure described previously, BMD (g/cm^2) and BMC (g) of the entire femur and tibia were determined with a PIXImus densitometer (version 1.4; GE Medical System Lunar) [14–16]. The BMD and BMC data are shown as mean ± standard deviation. Note that the "percent alterations" were defined as differences between the treated (T) and the contralateral (C) hindlimbs, such as [($T - C)/C \times 100$ (in %)].

Histological analysis of the growth plate

For histological sections, the isolated tibiae were fixed in 10% neutral buffered formalin. After measuring the length, weight, BMD, and BMC, the samples were decalcified in 10% ethylenediaminetetraacetic acid (EDTA) for 2 weeks, dehydrated in graded alcohols, and embedded in paraffin. Longitudinal sections were cut at a thickness of 7 μm and stained with hematoxylin and eosin (H&E). Histological measurements were conducted in the proximal tibia growth

plate using a Nikon Optiphot microscope (Nikon, Garden City, NY, USA) and a Bioquant digitizing system (R & M Biometrics, Nashville, TN, USA).

The height of the growth plate (GP) was defined from the apical border of the reserve zone to the lower border of the mineralized cartilage [17, 18]. The height of the hypertrophic zone (HZ) and of the proliferative zone (PZ) was also measured. Note that the upper boundary of the hypertrophic zone was identified at the margin of the chondrocytes that increased their size relative to those in the proliferative zone, whereas its lower boundary was at the terminal intact chondrocytes next to the metaphysis [19]. Heights were measured at 50 different locations in each section, and the height ratios (PZ/GP and HZ/GP) were calculated [20–22]. The cross-sectional areas of the growth plate, the proliferative zone, and the hypertrophic zone were also determined [23].

At the cellular level, the numbers of proliferative and hypertrophic chondrocytes were counted and the total number of chondrocytes was calculated as their sum. The height of hypertrophic chondrocytes was determined using at least 20 cells in each slice [19, 21].

Statistical analysis

The data were expressed as mean $\pm$ SD. One-way analysis of variance (ANOVA) was employed to evaluate statistical significance among the loaded and age-matched groups. For pairwise comparisons, a post hoc test was conducted using Fisher's protected least significant difference test. A paired t test was used to evaluate statistical significance between the treated and control samples in each group. All comparisons were two tailed, and statistical significance was assumed for $P < 0.05$.

Fig. 2 Load-driven alterations in bone length and bone weight. Three groups are the age-matched, contralateral (right limb), and loaded (left limb) groups. The single, double, and triple *asterisks* indicate $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively. **a** Alterations in femur length. **b** Alterations in femur weight. **c** Alterations in tibia length. **d** Alterations in tibia weight
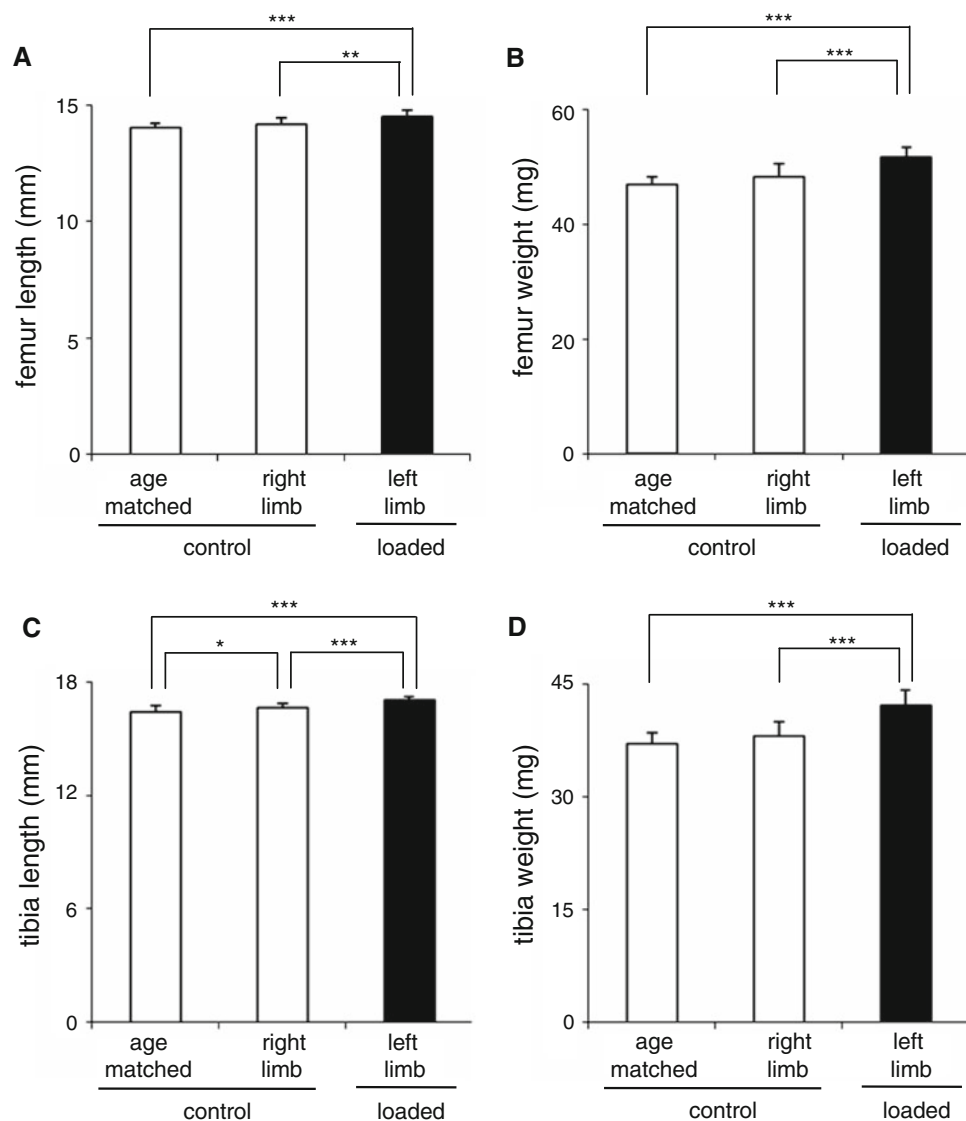
Results

Mouse characteristics

During knee loading, no apparent damage was detected at the site of loading or injection. Similarly, after treatment no mouse showed weight loss or diminished food intake.

Knee loading-driven elevation in bone length and bone weight in the femur and the tibia

Compared to the contralateral control, knee loading lengthened both the femur and the tibia (Fig. 2). First, the longitudinal length of the femur was increased by 2.3% (14.19 ± 0.28 mm in contralateral control; 14.51 ± 0.28 mm in knee loading) ($P < 0.01$) and the femoral weight was increased by 7.1% ($P < 0.001$). Second, the longitudinal length of the tibia was increased by 2.3% (16.68 ± 0.23 mm in contralateral control; 17.06 ± 0.21 mm in knee loading) ($P < 0.001$) and the tibia weight

was increased by 10.4% ($P < 0.001$). Compared to the age-matched control, knee loading increased the longitudinal length by 3.5% in the femur ($P < 0.001$) and by 3.7% in the tibia ($P < 0.001$). Furthermore, femur weight was increased by 10.2% ($P < 0.001$) and tibia weight by 13.9% ($P < 0.001$). No statistically significant difference was observed between the left and the right hindlimbs in the age-matched control group in bone length ($P = 0.92$ for the femur; $P = 0.97$ for the tibia) or bone weight ($P = 0.42$ for the femur; $P = 0.68$ for the tibia).

Knee loading-driven elevation of BMD and BMC in the femur and the tibia

Evaluation of bone quality with PIXImus revealed increase of BMD and BMC with knee loading (Fig. 3). Compared to the contralateral control, BMD in the femur was increased (0.0446 ± 0.0014 g/cm^2 in contralateral control; 0.0460 ± 0.0021 g/cm^2 in knee loading) ($P < 0.05$), and BMC in the femur was also elevated (0.0165 ± 0.0008 g in contralateral

Fig. 3 Load-driven alterations in bone mineral density (BMD) and bone mineral content (BMC). The groups are the same as in Fig. 2. The single, double, and triple *asterisks* indicate $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively. **a** Alterations in femur BMD. **b** Alterations in femur BMC. **c** Alterations in tibia BMD. **d** Alterations in tibia BMC
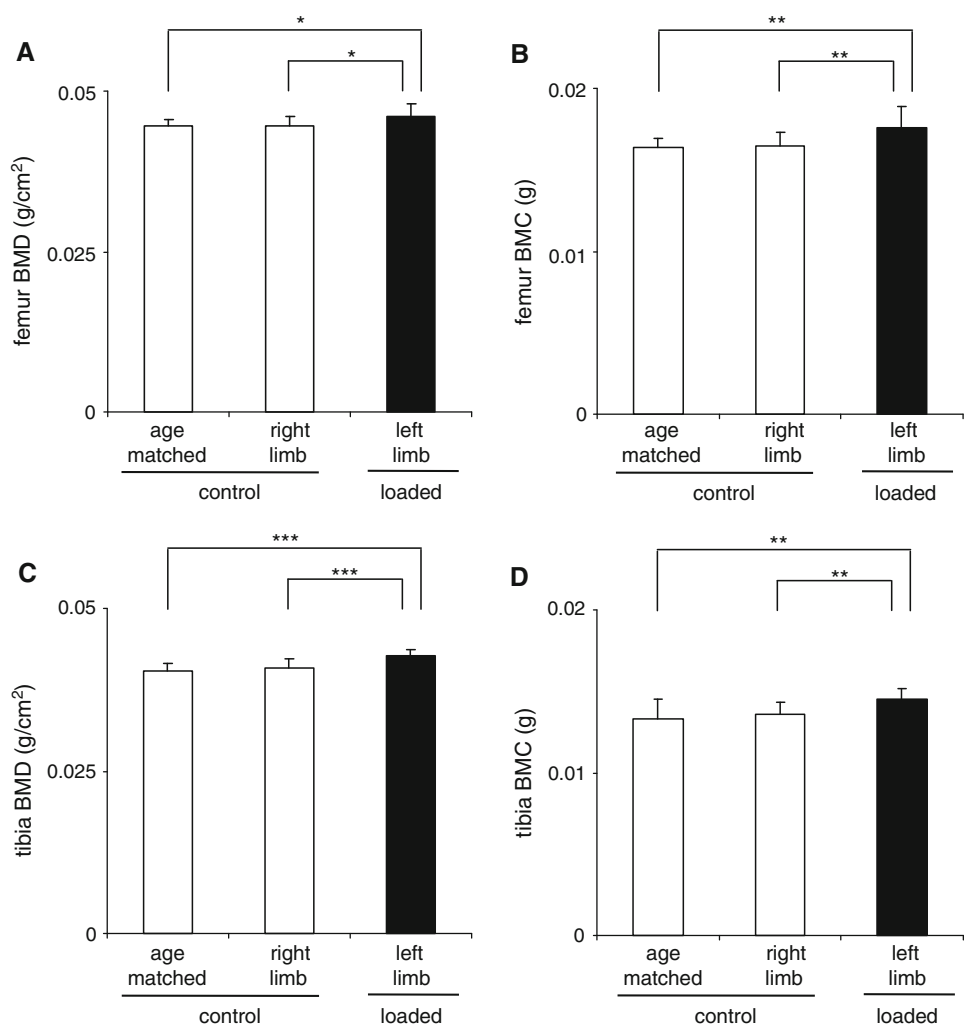
Fig. 4 H&E-stained sections of the growth plate in the proximal tibia. **a** Growth plate of the contralateral control. The *bracket* denotes the growth plate. **b** Growth plate (*bracket*) of the loaded tibia. **c** Proliferative and hypertrophic zones of the contralateral control. **d** Proliferative and hypertrophic zones of the loaded tibia. *Bars* **a**, **b** 100 μm; **c**, **d** 200 μm
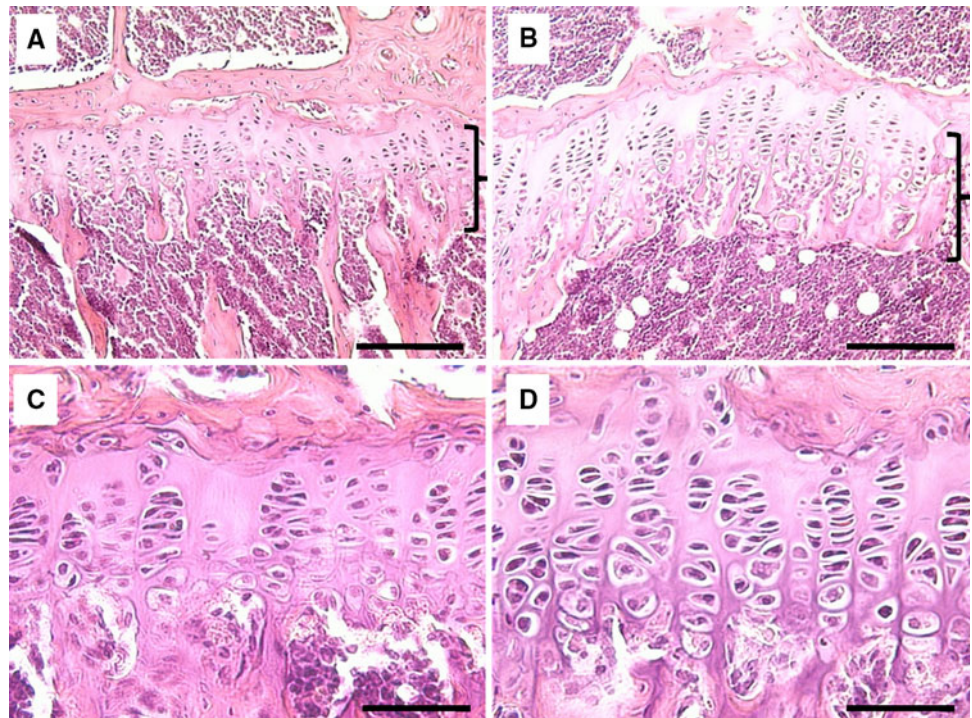
control; 0.0176 ± 0.0013 g in knee loading) ($P < 0.01$). Furthermore, BMD in the tibia was increased (0.0409 ± 0.0013 g/cm^2 in contralateral control; 0.0427 ± 0.0010 g/cm^2 in knee loading) ($P < 0.001$), with elevation in BMC (0.0136 ± 0.0007 g in contralateral control; 0.0145 ± 0.0007 g in knee loading) ($P < 0.01$). Compared to the age-matched control group, BMD was increased in femur ($P < 0.05$) and tibia ($P < 0.001$), with an increase in BMC in femur ($P < 0.01$) and tibia ($P < 0.01$). No statistically significant difference was detected between left and right hindlimbs in the age-matched control group in BMD ($P = 0.60$ for the femur; $P = 0.93$ for the tibia) or BMC ($P = 0.60$ for the femur; $P = 0.66$ for the tibia).
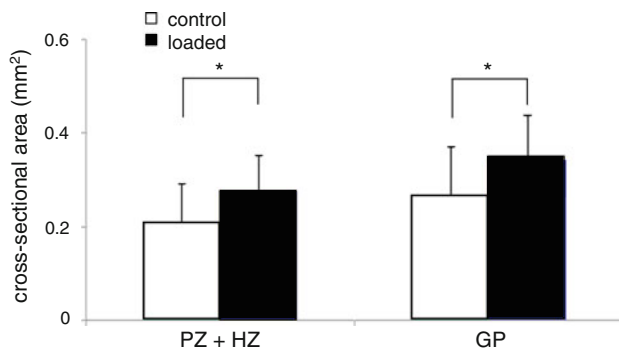
Increase in the height and the cross-sectional area of the growth plate

Histological analysis revealed that knee loading increased the height and the cross-sectional area of the growth plate in the proximal tibia (Fig. 4). First, the total growth plate height was increased by 19.5% (175 ± 25.6 μm in contralateral control; 210 ± 18.1 μm in loading) ($P < 0.001$), including the heights of the proliferative zone and the hypertrophic zone (Fig. 5a). In particular, the height of the hypertrophic zone was extended by 33.6% (48 ± 4.6 μm in contralateral control; 65 ± 3.4 μm in knee loading) ($P < 0.001$). Note that the height ratio of the hypertrophic zone to the growth plate (HZ/GP) was significantly increased ($P < 0.01$), whereas the ratio for the proliferative zone (PZ/GP) was not altered (Fig. 5b). Thus, the
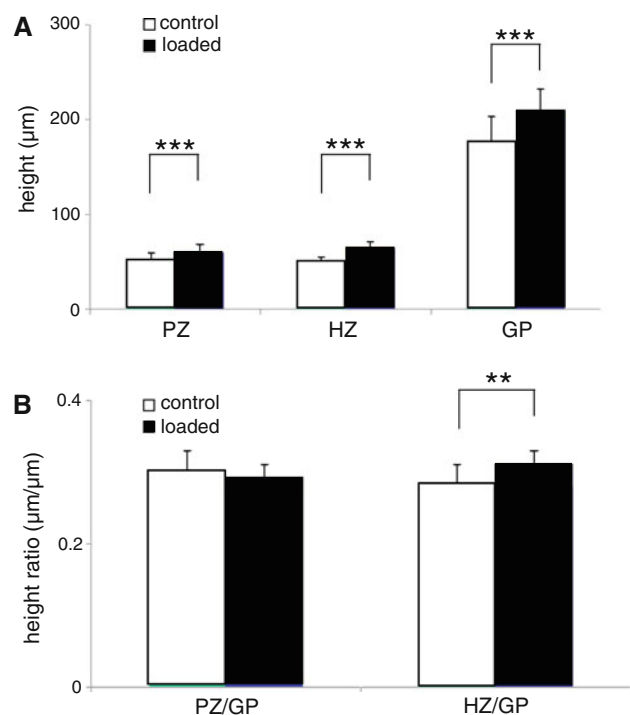
Fig. 5 Load-driven alterations in the height of the tibial growth plate. The double and triple *asterisks* indicate $P < 0.01$ and $P < 0.001$, respectively. *PZ* proliferative zone, *HZ* hypertrophic zone, *GP* total growth plate. **a** Height of the growth plate. **b** Height ratios defined as PZ/GP and HZ/GP

contribution of the hypertrophic zone for bone lengthening was more significant than that of the proliferative zone. Second, the cross-sectional area of the growth plate was

Fig. 6 Load-driven increase in the cross-sectional areas of the tibial growth plate. *PZ + HZ* proliferative and hypertrophic zones, *GP* total growth plate

increased by 30.7% (0.263 ± 0.108 mm^2 in contralateral control; 0.344 ± 0.095 mm^2 in knee loading) ($P < 0.05$) (Fig. 6). The areas of the proliferative and the hypertrophic zones were also enlarged ($P < 0.05$).

Alterations in the number and the height of chondrocytes in the growth plate

At the cellular level, the numbers of chondrocytes were increased in the total growth plate and the hypertrophic zone by 28.5% ($P < 0.05$) and 46.3% ($P < 0.01$), respectively (Fig. 7a). In the proliferative zone, however, no statistically significant difference in the numbers of cells was detected ($P = 0.053$). The ratio of the number of chondrocytes in the hypertrophic zone, defined as HZ/(PZ + HZ), was significantly increased, whereas the ratio in the proliferative zone as PZ/(PZ + HZ) was decreased (Fig. 7b). As in the results for height measurements, this observation for cell numbers indicates that the contribution of the hypertrophic zone is greater than that in the proliferative zone for bone lengthening. Last, the height of individual chondrocytes in the hypertrophic zone was elevated in the loaded side (16.3 ± 1.67 μm) compared to the control side (13.0 ± 1.45 μm; $P < 0.001$).

Discussion

This study demonstrated that laterally applied mechanical loads (knee loading) are able to lengthen the femur and the tibia. For lengthening bone, distraction osteogenesis is a widely used surgical procedure [24, 25]. In that procedure, a surgical cut is made in the bone to be lengthened, and the two sections of the bone are spread and fixed with an internal metal device and an external fixator. In spite of efficacy of this method, potential complications include delayed union resulting from slow healing and premature consolidation, in which the two sections fuse prematurely
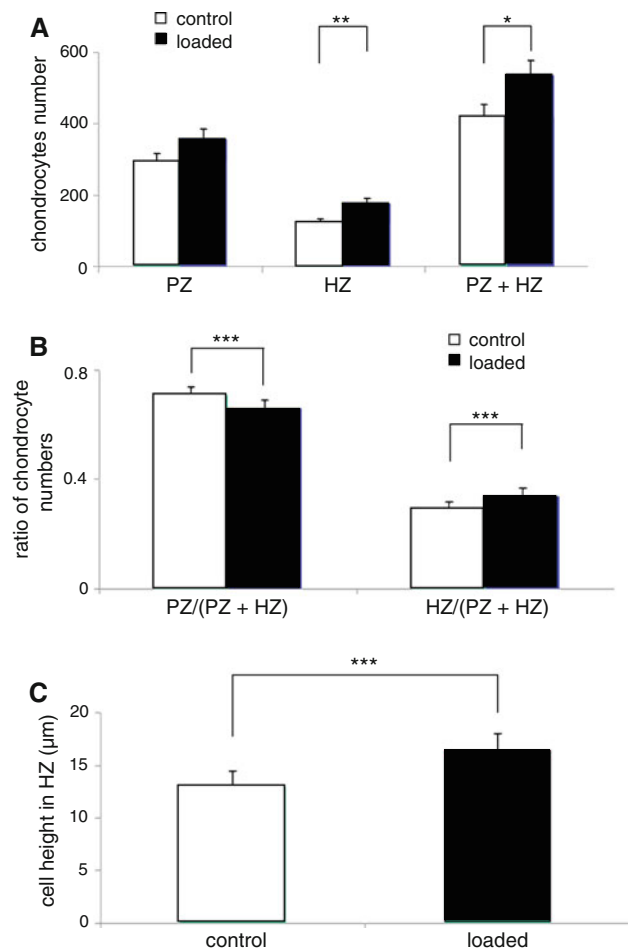
Fig. 7 Load-driven changes in the cellular level. The single, double, and triple *asterisks* indicate $P < 0.05$, $P < 0.001$, and $P < 0.001$, respectively. *PZ* proliferative zone, *HZ* hypertrophic zone. **a** Load-driven increase in the number of chondrocytes. **b** Ratios of the numbers of chondrocytes, defined as PZ/(PZ + HZ) and HZ/(PZ + HZ). **c** Increase in the height of chondrocytes in the hypertrophic zone

without effective lengthening and the bone must be cut again. Thus, the data presented herein with knee loading support a possibility of noninvasive treatments as an alternative to distraction osteogenesis.

Many studies have shown that various mechanical loading modalities stimulate anabolic responses and accelerate healing of bone damage. Little is known, however, about load-driven bone lengthening [26, 27]. The current study presents evidence that oscillatory loads laterally applied to the knee not only induce anabolic responses but also lengthen the femur and the tibia. According to our molecular analysis with microarray-derived whole-genome mRNA expression profiles and quantitative real-time polymerase chain reaction (PCR), molecules in phosphatidylinositol 3-kinase (PI3K), extracellular matrix (ECM)–receptor interactions, transforming growth factor-beta (TGF-β) signaling, and Wnt signaling were involved in

the joint-loading driven responses [28–30]. Interestingly, it has been reported that the PI3K pathway is also activated in a human chondrocyte culture system in response to administration of insulin-like growth factor (IGF) [31].

Our histological analysis revealed that, compared to the contralateral controls, knee loading lengthened the growth plate, including the lengths of the proliferative and hypertrophic zones. The total length increase in the growth plate was more than the sum of the increases in the proliferative and hypertrophic zones, indicating that other regions such as the resting and calcifying zones were also affected [18]. Because the cross-sectional area of the growth plate is significantly increased with knee loading, the data support that the bone-lengthening effects are not limited only to the lateral or medial loading site. At the cellular level, the number of chondrocytes in the hypertrophic zone was increased together with their cellular height. Our results are consistent with the notion that dynamic tensile and compressive loads stimulate and suppress longitudinal growth, respectively [32, 33].

In knee loading, the rate of lengthening with 0.5 N loads (peak-to-peak) was 0.1% per bout (femur) and 0.1% per bout (tibia) for 5-min loading per day. It is important to evaluate whether the observed rate of lengthening is kept consistent throughout a longer treatment period than that in the present procedure. Interestingly, in vivo ulna loading studies with rats exhibited adaptive responses not only in loaded but also unloaded contralateral bones [34]. The present loading study also showed that compared to age-matched controls, both loaded and contralateral hindlimbs increased in length in the tibia. Future studies will provide a better understanding of the observed systemic effects in response to joint loading.

In summary, with knee loading, bone-lengthening effects are observed in the femur and tibia. Further studies for understanding the mechanism and efficacy behind bone lengthening with knee loading are needed to develop a potential therapy for treating limb length discrepancy.

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