

## **Upregulation of osteogenic factors induced by high-impact jumping suppresses adipogenesis in marrow but not adipogenic transcription factors in rat tibiae.**

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### **Abstract**

Jump training is a high-impact training regimen that increases bone volume in young bones. The aim of our study was to determine whether downregulation of adipogenesis that is associated with upregulation of osteogenesis is detected after jump training in growing rat tibiae. Four week-old rats were jump-trained for 1, 2, or 4 weeks for 5 days/week, and the height of jumping progressively increased to 35 cm. We performed morphometry to directly quantitate changes in bone volume and marrow adipocyte distribution in tibiae after the jump training. We also examined changes in expression of osteogenic and adipogenic transcription factor proteins and mRNAs after the jump training. Four weeks of jump training induced an increase in trabecular bone volume, which was associated with recruitment of runt-related transcription factor 2-expressing cells, as well as a decrease in marrow fat volume. However, peroxisome proliferator-activated receptor (PPAR)  $\gamma$ 2 protein and mRNA expression levels did not change following high-impact jump training. The mRNA expression levels of the adipocyte differentiation genes CCAAT/enhancer-binding proteins (C/EBP)  $\alpha$ , C/EBP $\beta$ , and C/EBP $\delta$  also showed no change during the training period in jump-trained rats. We suggest that the levels of osteogenic factors that were upregulated by mechanical loading from high-impact jumping suppress adipogenesis in marrow rather than adipogenic transcription factors.

**Key words:** Jump training, Mechanical loading, Pluripotent stem cell, Osteoblast, Adipocyte

## Introduction

Increased bone volume due to exercise is related to the influence of mechanical loading, which is elicited by ground reaction forces and muscle contraction, rather than exercise intensity expressed as maximum oxygen consumption (Heinrich et al. 1990; Risser et al. 1990; Umemura et al. 1995). Athletes performing high-impact exercise (i.e., volleyball and basketball) have greater bone density than those who do low-impact exercises (i.e., swimming) and non-exercising controls (Risser et al. 1990).

Bone volume and marrow fat volume are inversely proportional (Morrie 1985; Justesen et al. 2001; Moerman et al. 2004; Benayahu et al. 2000; Ahdjoudj et al. 2002, 2005; Botolin et al. 2005). Osteoporosis and age-related osteopenia are associated with an increase in marrow fat volume (Morrie 1985; Justesen et al. 2001; Moerman et al. 2004). Various osteopenic animals have also shown an increase in marrow fat volume (Benayahu et al. 2000; Ahdjoudj et al. 2002, 2005; Botolin et al. 2005). Conversely, osteogenic physical exercise decreases marrow fat volume (David et al. 2007; Menuki et al. 2008).

Osteoblasts and marrow adipocytes originate from marrow multipotent stem cells (Pittenger et al. 1999; Chamberlain et al. 2007). The differentiation programs of pluripotent stem cells are distinctly controlled by the coordinated regulation of osteogenic and adipogenic transcription factors. Osteoblastic differentiation is regulated by runt-related transcription factor 2 (Runx2), followed by SP7 (also known as osterix) (Otto et al. 1997; Komori et al. 1997; Nakashima et al. 2002; Komori 2005). Runx2 promotes expression of bone matrix protein genes and plays a role in osteoblastic differentiation (Otto et al. 1997; Komori et al. 1997; Komori 2005). The role of osterix is not clear, but appears to be related to the maturity of osteoblasts (Nakashima et al. 2002; Komori 2005). Adipogenic differentiation is driven by members of the CCAAT/enhancer binding protein (C/EBP) family, including C/EBP $\alpha$ ,  $\beta$ , and  $\delta$ , and peroxisome proliferator-activated receptor  $\gamma$ 2 (PPAR $\gamma$ 2) (Mandrup and Lane 1997; Gregoire et al. 1998). C/EBP $\beta$  and  $\delta$  induce the expression of C/EBP $\alpha$  and PPAR $\gamma$ 2. C/EBP $\alpha$  and PPAR $\gamma$ 2 regulate adipocyte-specific gene expression and play a role in the formation of mature lipid-filled adipose cells from stem cells (Rosen 2005).

Mechanical loading appears to increase osteogenic transcription factor expression and decrease adipogenic transcription factor expression (David et al. 2007; Sen et al. 2008; Luu et al. 2009). Mechanical loading of the mesenchymal stem cell line C3H10T1/2 increases Runx2 expression and decreases PPAR $\gamma$ 2 expression, leading to the

promotion of osteoblastic differentiation and inhibition of adipogenic differentiation (David et al. 2007; Sen et al. 2008). Whole-body vibrations in mice increase expression levels of Runx2 mRNA and decrease expression levels of PPAR $\gamma$ 2 mRNA in marrow cells (Luu et al. 2009).

Although high-impact exercise to increase bone volume may strongly inhibit adipogenic differentiation, there are no specific data showing that high-impact exercise suppresses the adipogenic differentiation that is associated with promotion of osteoblastic differentiation from marrow pluripotent stem cells.

In animal studies, jump training is a high-impact training regimen that increases bone mass more than aerobic running training (Umemura et al. 1995; Notomi et al. 2000). Fat-free dry weights of the femur and the tibia are significantly greater in jump-trained rats than in run-trained rats (Umemura et al. 1995). The mass and strength of the lumbar vertebrae and of the mid-diaphysis of the femur are significantly greater in jump-trained rats than in aerobic running-trained rats (Notomi et al. 2000). Jump training is effective during recovery from osteopenia induced by ovariectomy (OVX) or tail suspension in rats (Honda et al. 2001; Ju et al. 2008). Jump training significantly increases the fat-free dry weight, periosteal perimeter, and cortical area in OVX rat tibiae (Honda et al. 2001). Jump training increases trabecular thickness and number, as well as connectivity in tail-suspended rat femurs (Ju et al. 2008). We used jump training to examine the effects of mechanical loading on the differentiation of marrow pluripotent stem cells. The aim of our study was to determine whether downregulation of adipogenesis that is associated with upregulation of osteogenesis is detected after high-impact exercise training. We performed morphometry to directly quantitate changes in bone volume and marrow adipocyte distribution in the tibiae after jump training. Using immunohistochemistry and polymerase chain reaction, we also examined changes in expression of osteogenic and adipogenic proteins and mRNA after jump training.

## **Methods**

### Experimental animals

Thirty-one Fischer 344 strain female rats (4 weeks old) were divided into two groups: an age-matched handling

control group (Cont; n = 5 per time point) and a jump training group (Jump). The rats in the Jump group were trained for 1, 2, or 4 weeks (n = 5 or 6 per time point; see Table 1). The rats were housed in groups of two per cage in the animal facility under a 12:12-h light-dark cycle at room temperature ( $23 \pm 2$  °C) and  $55 \pm 5\%$  humidity. The rats were maintained on a diet of rodent chow (CE-2, CLEA Japan, Tokyo, Japan) and water *ad libitum*. All animal experiments were performed in accordance with the guidelines presented in *Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences*, published by the Physiological Society of Japan.

### Jump training

The rats in the Jump group were individually placed at the bottom square of a board box (Umemura et al. 1995, 1997). They jumped following an electric stimulus and grasped the top of the board with their forelimbs and then climbed up the board. They were then repositioned by hand at the bottom of the box in preparation for the next jump. To avoid causing mechanical loading to the rat tibiae during landing impact, we gently repositioned the rats by hand. In this manner, we were able to study only the influence of jumping and not of landing. As the rats became accustomed to jump training at approximately 3 days, the electric stimulus was no longer needed. The jump-trained rats were trained for 1, 2, and 4 weeks at a jumping rate of 20 repetitions per day for 5 days/week. The jump training began with an initial jump height of 20 cm, and thereafter the height was progressively increased to 35 cm by the fourth week. Because we wanted to determine the exact number of strains per day to the bones, failed trials in which the rats could not climb up were also counted. The time required per jump was about 5 s.

After the 4-week training program, we extracted the tibiae and hind-limb skeletal muscles from Cont and Jump animals. We assessed the effect of jump training on bone volume and marrow fat volume using histochemical and immunohistochemical techniques. Additionally, RNA was extracted from tibiae for quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR) analysis after jump training programs of 1, 2, and 4 weeks.

### Histochemistry and immunohistochemistry

The tibiae were quickly frozen in isopentane and cooled using liquid nitrogen for carboxymethyl cellulose (CMC) gel block preparation. The frozen tibiae were re-embedded in 5% CMC gel and frozen. Each CMC block was covered

with a polyvinylidene chloride film coated with synthetic rubber cement. Serial longitudinal sections (4 and 20  $\mu\text{m}$  thickness) with the film were cut using a cryostat (CM3050S, Leica, Wetzlar, Germany) with a disposable tungsten carbide blade (TC-65, Leica) at  $-25\text{ }^{\circ}\text{C}$  (Kawamoto and Shimizu 2000).

Trabecular bone architecture was visualized with Alizarin red staining on 4- $\mu\text{m}$ -thick sections. Freeze-dried sections were fixed with 3.7% formaldehyde for 15 min and rinsed with deionized water. The sections were incubated with 1% Alizarin red S (Nacalai Tesque, Kyoto, Japan) solution for 1 min and rinsed with 3% KOH. The sections were mounted with water-soluble mounting medium (Mount-Quick Aqueous, Daido Sangyo, Saitama, Japan). The lipid droplets in bone marrow were assessed by Oil-red O staining of the 20- $\mu\text{m}$ -thick sections. Briefly, freeze-dried sections were fixed with 3.7% formaldehyde for 15 min and rinsed with deionized water. The sections were incubated with Oil-red O solution for 5 min and rinsed with deionized water. The sections were mounted with water-soluble mounting medium. The diameters of lipid droplets that were visualized following Oil-red O staining were 4-59  $\mu\text{m}$ .

For immunohistochemical analysis, the sections were fixed with 4% paraformaldehyde for 10 min and then washed with 0.1 M phosphate-buffered saline (PBS). To quench endogenous peroxidase activity, the sections were incubated with a solution of methanol containing 0.6%  $\text{H}_2\text{O}_2$  for 15 min. The sections were washed with 0.1 M PBS, blocked with 0.1 M PBS containing 2% bovine serum albumin, 1.5% normal rabbit serum, and 0.1% Triton X-100, and then incubated for 30 min at room temperature with goat anti-Runx2 antibody (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or goat anti-PPAR $\gamma$ 2 antibody (1:200; Santa Cruz Biotechnology) in 0.1 M PBS containing 2% bovine serum albumin and 0.1% Triton X-100. Then the sections were washed in 0.1 M PBS and incubated with secondary antibodies: horseradish peroxidase (HRP)-conjugated (1:250; Molecular Probes, Eugene, OR, USA) or biotinylated anti-goat IgG from the Vectastain ABC kit (1:200; Vector Labs, Burlingame, CA, USA). HRP-conjugated secondary antibodies were used on sections that were incubated with the Runx2 antibody. Biotinylated secondary antibodies were used on sections that were incubated with the PPAR $\gamma$ 2 antibody. The sections were then washed in 0.1 M PBS. Sections that were incubated with HRP-conjugated secondary antibodies were visualized with diaminobenzidine (DAB) solution. The immunoreactive signals of sections that were incubated with biotinylated secondary antibody were enhanced with the *avidin-biotin complex*, and then incubated with DAB solution. Nuclei were counter-stained with methyl green or

hematoxylin.

Quantitative analysis of microscopic observations was performed using a digital microscope (Coolscope, Nikon, Tokyo, Japan), which comprised a video monitor and a personal computer with image analysis software (ImageJ ver. 1.38, <http://rsb.info.nih.gov/ij/>). We performed morphometry in the central region of the tibial metaphysis (Figure 1). Trabecular bone measurements and Runx2-expressing cell counts were performed on a 4.4-mm<sup>2</sup> area (equal to the sum of Regions II and III in Figure 1), 1.3 mm distal from the growth plate-cancellous bone junction of the proximal tibia to exclude the primary cancellous bone (Ke et al. 1992). The ratios of Runx2-expressing cell numbers per millimeter of trabecular bone surface (mm) were determined. Within the tibiae, marrow conversion in the proximal metaphyses occurs last (Morrie 1985). Thus, marrow adipocyte measurements were performed at the central part of proximal metaphyseal regions ranging from the growth plate to 4 mm distal. Moreover, features of marrow conversion in the proximal metaphysis of tibiae may be different in every region. We divided the tibial proximal metaphysis into three regions (Figure 1), based on features of the cancellous bone structure, and assessed the lipid droplet distribution and the number of PPAR $\gamma$ 2-expressing cells. Each analysis region was a 2.2-mm<sup>2</sup> visual field taken using a microscope (5 $\times$  magnification). Region I was located right under the growth plate-cancellous bone junction (from the most distalis-sided hypertrophy chondrocytes to 1.3 mm distal). We observed primary-secondary cancellous bone junctions. Region II was located in the middle of a secondary cancellous bone region (from 1.3 to 2.6 mm under the growth plate). Region III was located at the end of a secondary cancellous bone region (from 2.6 to 3.9 mm under the growth plate). The lipid droplets and PPAR $\gamma$ 2-expressing cells that overlapped with borders of each measurement region were not counted. There were no differences in the marrow areas of each analysis region between the Cont and Jump groups. The numbers of lipid droplets and PPAR $\gamma$ 2-expressing cells, identified by Oil-red O and PPAR $\gamma$ 2 antibodies, were counted per square millimeter of marrow area (mm<sup>2</sup>).

#### RNA extraction and cDNA synthesis

Bone marrow cells from the rat tibiae were flushed out from the proximal metaphysis (Ahdjoudj et al. 2002, 2005). Total RNA was extracted using an RNeasy mini kit (Qiagen, Tokyo, Japan). The total RNA concentration was quantified

by measuring the absorbance at 260 nm (BioPhotometer plus, Eppendorf, Tokyo, Japan). All samples had an absorbance ratio (260 nm/280 nm) of at least 1.9. First-strand cDNA was reverse transcribed from total RNA (1 µg) using a QuantiTect Rev. Transcription kit (Qiagen). The cDNA samples were aliquoted and stored at -80 °C.

#### Real-time RT-PCR analysis

Quantitative PCR analysis was performed using Opticon™ DNA Engine (MJ Research, Waltham, MA, USA) according to the manufacturer's instructions. Quantitative PCR reactions for the genes examined were performed in 25 µl with 50 ng cDNA, 2.5-5.0 µl of primers, and 12.5 µl 2× QuantiFast SYBR Green PCR Master Mix (Qiagen). The primers we used for the genes we examined are as follows: bone morphogenetic protein (BMP)-2, 5'-GGAAACTTCCCGACGCTTCT-3' (sense) and 5'-CCTGCATTTGTTCCCGAAA-3' (antisense); BMP-4, 5'-TTATGAGGTTATGAAGCCCCCA-3' (sense) and 5'-GCTCACATCGAAAGTTTCCCAC-3' (antisense); Runx2, QT01620647 (Qiagen); osterix (SP7), QT01083082 (Qiagen); PPARγ2, 5'-CGCTGATGCACTGCCTATGA-3' (sense) and 5'-GGGCCAGAATGGCATCTCT-3' (antisense); C/EBPα, QT00395010 (Qiagen); C/EBPβ, QT00366478 (Qiagen); C/EBPδ, QT00368599 (Qiagen); and β2-microglobulin (β2M), QT00176295 (Qiagen). β2M served as an internal control. Primers for BMP-2, BMP-4, and PPARγ2 were obtained from Invitrogen (Tokyo, Japan). Amplification conditions were as follows: an initial 5 min at 95 °C, 40 cycles of denaturation at 95 °C for 10 s, annealing and extension at 65 °C for 30 s. PCR was performed independently for each animal of a five- or six-member group in a volume of 25 µl using 96-well optical-grade PCR plates and optical strip caps (Bio-Rad, Tokyo, Japan).

#### Statistical analysis

The results are shown as the means ± standard deviation (SD). Differences between groups were examined using Student's *t*-test (bone morphometry and Runx2-expressing cell counts). Lipid droplet data and numbers of PPARγ2-expressing cells were assessed with a two-way analysis of variance (ANOVA) for the training and the different regions of the marrow. Body weight, muscle weight, and mRNA expression data were assessed with two-way ANOVA for the time and training, followed by the Scheffe's test or Student's *t*-test. P values of < 0.05 were considered significant.

## Results

### Body and hind-limb muscle weight

Body weights increased during development ( $p < 0.05$ , Table 1). Hind-limb muscle weights in 5-week-old rats were significantly higher than in 4-week-old rats ( $p < 0.05$ , data not shown). Similarly, hind-limb muscle weights in 8-week-old rats were significantly higher than in 4- and 5-week-old rats ( $p < 0.05$ , data not shown). However, the body and hind-limb muscle weights were not changed by jump training.

### Bone morphometry

We assessed the jump training effects on bone architecture and mass using morphometric analyses. Tibia bone wet weight did not change following 4 weeks of jump training (Cont,  $303.2 \pm 14.2$  mg; Jump,  $307.1 \pm 14.3$  mg). There were no differences in the tibia length (Cont,  $30.8 \pm 0.4$  mm; Jump,  $30.6 \pm 0.4$  mm) or the periosteal perimeter of the tibia-fibula junction (Cont,  $7.3 \pm 0.3$  mm; Jump,  $7.4 \pm 0.2$  mm) between the Jump and Cont groups. We evaluated trabecular bone thickness and areas using histomorphometric analyses. The mean trabecular bone thickness (Cont,  $46.4 \pm 5.3$   $\mu$ m; Jump,  $45.7 \pm 4.4$   $\mu$ m) did not change following 4 weeks of jump training. Jump training significantly ( $p < 0.05$ ) increased trabecular bone area by 25% (Figure 2).

In this study, we did not directly assess trabecular number. However, from the analysis of trabecular bone area and trabecular bone thickness, it is possible that an increase in trabecular number occurred.

### Runx2 Immunohistochemistry

Using histological sections, we observed an increase in trabecular bone volume in the proximal metaphysis of tibiae following jump training. The transcription factor Runx2 plays an important role in the regulation of osteoblast function and differentiation. We evaluated Runx2 protein expression using immunohistochemistry. Runx2-expressing cells at the bone surfaces of proximal tibiae were observed in Cont and Jump rats (Figure 3A and B). Runx2-expressing cells were cuboidal or spindle-shaped. Cuboidal Runx2-expressing cells were located on the bone surface. Spindle-shaped Runx2-expressing cells were located on the bone surface and in marrow stroma, which was slightly displaced from the bone surface (Figure 3B, arrows).

The secondary cancellous bones were located in Regions II and III (Figure 1). The number of Runx2-expressing cells on the cancellous bone surface was significantly ( $p < 0.05$ ) increased following 4 weeks of jump training (Figure



3C).

#### Distribution of lipid droplets

We visualized lipid droplets in marrow adipocytes using Oil-red O staining of non-decalcified frozen sections (20  $\mu\text{m}$  thick). Oil-red O stains neutral lipids (mainly triglycerides) red. Bones are not stained by Oil-red O and appear gray. We observed that lipid droplets were uniformly distributed within the proximal metaphyseal marrow stroma of Cont and Jump rat tibiae (Figure 4A and B). Several lipid droplets were observed in marrow stroma among the primary cancellous bones in Cont and Jump rats. Most lipid droplets were spherical or had oblate spherical shapes (inset images, Figure 4A and B). Features of marrow conversion in the proximal metaphysis of tibiae may be different in every region. We divided tibial proximal metaphysis into three regions, based on features of the cancellous bone structure, and assessed the lipid droplet distribution. There were no differences in the numbers of lipid droplets between Regions II and III in the same group of rats. The number of lipid droplets in Region I, however, was significantly ( $p < 0.05$ ) smaller than those in Regions II and III in the same group of rats. Region I is located right under the growth plate-cancellous bone junction, at the most proximal side of the proximal metaphyses. The distances between trabeculae in the primary cancellous bone region are narrow. These histological features appeared to confine many of the lipid droplets to Region I. Although the number of lipid droplets in Region III (near the diaphyses) did not change following jump training, the numbers in Regions I and II were decreased following 4 weeks of jump training ( $p < 0.05$ , Figure 4C).

#### Immunohistochemistry for PPAR $\gamma$ 2

Four weeks of jump training decreased the number of lipid droplets in the proximal metaphysis of tibiae. Transcription factor PPAR $\gamma$ 2 plays an important role in the regulation of adipogenesis. We evaluated PPAR $\gamma$ 2 protein expression using immunohistochemistry in the proximal metaphysis of tibiae.

We observed that PPAR $\gamma$ 2-expressing cells were uniformly distributed within the metaphyseal marrow stroma, similar to the distribution of lipid droplets, in Cont and Jump rat tibiae (data not shown). There were no differences in the number of PPAR $\gamma$ 2-expressing cells between each measurement region in the same group. Although the number of lipid droplets decreased with jump training, the number of PPAR $\gamma$ 2-expressing cells did not change with jump training (Figure 4D).

#### Quantification of mRNA expression

Expression levels of BMP mRNAs did not significantly change during development (data not shown). Transcription factor (Runx2, osterix, PPAR $\gamma$ 2, C/EBPs) mRNA expression levels in 8-week-old rats were significantly higher than in 4- and 5-week-old rats (data not shown,  $p < 0.05$ ). We evaluated the effects of jump training on the time course of mRNA expression of BMPs and osteogenic and adipogenic transcription factors in marrow cells using real-time RT-PCR analysis (Figure 5). BMP-2 mRNA expression levels did not significantly change during the training period. BMP-4 mRNA expression levels were significantly higher after 1 and 2 weeks of jump training than in each age-matched control group ( $p < 0.05$ ). Runx2 mRNA expression levels were significantly higher following 4 weeks of jump training than in the age-matched control group ( $p < 0.05$ ), whereas osterix mRNA expression levels were significantly higher following 2 weeks of jump training ( $p < 0.05$ ). No significant changes in PPAR $\gamma$ 2 or C/EBPs mRNA expression levels were observed between the Cont and Jump groups throughout the training period.

## **Discussion**

We have presented evidence that high-impact jump training induces an increase in trabecular bone volume and a decrease in marrow fat volume in the proximal metaphysis of tibiae of growing rats. These histological observations support the hypothesis that mechanical loading, which is elicited by jump training, plays an important role in osteogenesis-adipogenesis balance.

It is well known that mechanical loading from physical activity is important for skeletal development. Among the various types of exercise, high-impact exercise training is the most effective for increasing bone volume (Risser et al. 1990; Umemura et al. 1995; Notomi et al. 2000). An increase in trabecular bone volume in jump-trained rats is supported by the apparent recruitment of Runx2-expressing cells. Expression of Runx2 is induced primarily by BMPs (Ikegame et al. 2001; Komori 2005). Runx2, which is specifically expressed in osteoblast lineage cells, promotes production of bone matrix proteins such as type I collagen, osteocalcin, and osteopontin, and it plays a major role in osteoblastic differentiation (Komori et al. 1997; Komori 2005). Runx2 also inhibits the terminal differentiation of mature osteoblasts to resting cells (bone-lining cells) (Komori 2005). Runx2-expressing cells were increased in association with the upregulation of BMP-4 mRNA. Runx2-expressing cells were also observed on the bone surfaces and marrow stroma (not far from the bone surface). Runx2-expressing cells located on bone surfaces were either cuboidal or spindle-shaped.

Cuboidal cells appeared to be mature osteoblasts, and their presence indicated that synthesis of bone matrix proteins was active. Spindle-shaped cells located on bone surfaces appeared to be immature osteoblasts. The Runx2-expressing cells located in marrow stroma were spindle-shaped, indicating that they are pre-osteoblasts that had newly differentiated from pluripotent stem cells (Ikegame et al. 2001; Kondo et al. 2001).

BMP promotes not only Runx2 expression but also expression of osterix (Nakashima et al. 2002). In our study, BMP-4 mRNA expression levels were significantly higher after 1 and 2 weeks of jump training than in the control group. BMP mRNA expression increased immediately following mechanical loading, although it appeared to be a temporary increase (Ikegame et al. 2001; Menuki et al. 2008). Tensile stress increases BMP mRNA expression by 6 h in mouse calvaria and expression remains elevated for 48 h (Ikegame et al. 2001). Tower climbing exercise increases BMP mRNA expression by 4 days in mouse tibiae (Menuki et al. 2008). However, the expression level of BMP mRNA returns to the normal level in 7 days after the start of exercise (Menuki et al. 2008). Thus, a temporary increase in BMP mRNA expression levels following jump training is possible.

Adaptation of marrow adipocytes to mechanical loading elicited by jump training was observed in Regions I and II. Within the tibiae, marrow conversion to fat occurs first in the diaphyses, and then in the distal metaphyses, and finally in the proximal metaphyses (Morrie 1985). It is reasonable to hypothesize that numerous pluripotent stem cells exist in the regions where marrow conversion has not occurred. Region III is located near the diaphyses, mostly within the regions of analysis. Marrow conversion in Region III may have already been occurring at an early developmental stage (before 4 weeks of age when the jump training began).

Interestingly, downregulation of PPAR $\gamma$ 2 protein expression was not induced by high-impact jump training. PPAR $\gamma$ 2 and C/EBP $\alpha$ ,  $\beta$ , and  $\delta$  mRNA expression levels in bone marrow cells were also unchanged during the 4 weeks of the experimental period. However, jump training appeared to affect marrow adipocytes in Regions I and II. The differentiation programs of adipocytes are distinctly controlled by the coordinated regulation of PPAR $\gamma$ 2 and C/EBP family members. It has been reported that mechanical loading may suppress adipogenic differentiation by downregulating these adipogenic transcription factors in vitro and in vivo (David et al. 2007; Menuki et al. 2008; Sen et al. 2008; Luu et al. 2009). The mechanical loading elicited by high-impact jumping may not have influenced adipose lineage cells that exist in reticular marrow stroma, which is in contrast to in vitro studies that stimulated the cells directly.

Moreover, fluid flow and strain associated with mechanical loading on bone are detected in osteocytes that exist in the mineralized bone matrix (Duncan and Turner 1995). These stimuli are converted to biological signals in osteocytes, and then transmitted to other osteocytes and osteoblasts through the three-dimensional cellular network in the bone tissue formed by osteocytes and gap junctions (Jiang and Cherian 2003; Cherian et al. 2005). There are no specific data showing that adipose lineage cells located in marrow stroma connect the network formed by osteocytes and osteoblasts. These histological features may have caused the differences in the molecular response following high-impact jumping, including changes in osteogenic but not adipogenic transcription factor levels.

Several osteogenic cytokines suppress adipogenesis. Transcription factors that regulate stem cell differentiation also have reciprocal inhibitory functions, which may be associated with the decrease in marrow fat volume. Jump training strongly upregulated the expression of these osteogenic factors. Transforming growth factor (TGF)- $\beta$  inhibits the activity of PPAR $\gamma$  by promoting its phosphorylation (Hu et al. 1996; Adams et al. 1997; Ahdjoudj et al. 2005). BMPs, members of the TGF super-family, strongly inhibit adipogenic differentiation and promote osteoblastic differentiation (Wozney et al. 1988; Sen et al. 2008). In addition, mesenchymal stem cells from Runx2<sup>-/-</sup> mice differentiate into adipocytes in osteoblastic differentiation conditions; introduction of Runx2 prevents this aberrant adipocyte differentiation (Kobayashi et al. 2000; Enomoto et al. 2004).

We have presented evidence that high-impact jump training induces an increase in trabecular bone volume and a decrease in marrow fat volume in the proximal metaphysis of tibiae of growing rats. It is possible that growth supported these dramatic changes. In general, stem cells in young animals have a higher differentiation potential compared to stem cells in older animals (He et al. 2009). The environment that promotes differentiation from mesenchymal stem cells to osteoblasts is gradually lost with aging (Abdallah et al. 2006). It is not clear whether results of this study apply to osteopenia (induced by aging, tail-suspension, or OVX) in which fat is stored in the marrow (Benayahu et al. 2000; Justesen et al. 2001; Ahdjoudj et al. 2002, 2005; Moerman et al. 2004). However, jump training increases bone mass in aged, tail-suspended, and OVX rats (Umemura et al. 1995; Honda et al. 2001; Ju et al. 2008). Thus, jump training may decrease adipocytes in osteopenic bone in which fat is stored in the marrow.

## Conclusion

High-impact jump training induces an increase in bone volume and a decrease in fat volume in the proximal metaphysis of tibiae. Jump training recruits osteoblasts by upregulating the expression of Runx2, a mechanism by which the mechanical loading from high-impact jumping promotes osteoblastic differentiation. Molecular changes in adipogenic transcription factors were not observed, even though a decrease in marrow fat volume was induced by jump training. We suggest that expression of osteogenic factors, which are upregulated by the mechanical loading from high-impact jumping, suppresses adipogenesis in marrow rather than influencing adipogenic transcription factors.

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Conflicts of interest statement: None.

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## Legends

Fig. 1 Micrograph showing analysis regions (each 2.2 mm<sup>2</sup>) of the central metaphysis in sections of the proximal tibial metaphysis. Calcified bones were stained with alizarin.

Fig. 2 Effects of jump training on trabecular bone area in the proximal tibia. The ratios of trabecular bone area to the analysis area (4.4 mm<sup>2</sup>) in the Cont and Jump groups are shown. The trabecular analysis area equals the sum of Regions II and III in Figure 1. Data are the means  $\pm$  S.D. \*p < 0.05, compared with the Cont group.

Fig. 3 Light micrographs of Runx2-expressing cells in the Cont and Jump rat tibiae (A and B). Runx2-immunoreactive cells appear brown. Nuclei were stained with hematoxylin. Runx2-expressing cells were cuboidal and spindle-shaped. Cuboidal Runx2-expressing cells were located on the bone surface. Spindle-shaped Runx2-expressing cells were located on the bone surfaces, and those in the marrow stroma were slightly displaced from the bone surface. Cuboidal Runx2-expressing cells appear to be mature osteoblasts. Spindle-shaped Runx2-expressing cells appear to be immature osteoblasts or pre-osteoblasts (B, arrows). The ratios of these Runx2-expressing cell numbers to the length of the secondary cancellous bone surface in the Cont and Jump groups are shown (C). The secondary cancellous bones of a representative rat were located in Regions II and III as shown in Figure 1. Data are the means  $\pm$  S.D. \*p < 0.05, compared with the Cont group.

Fig. 4 Light micrographs showing longitudinal sections of the proximal tibia (Oil-red O staining, A and B). The lipid droplets (mainly neutral triglycerides) in marrow adipocytes were stained red with Oil-red O. Most of the lipid droplets appeared spheroid or oblate spheroid (inset images, A and B). The lipid droplets were observed in the marrow stroma in the proximal metaphyseal tibiae of Cont and Jump rats. The ratios of lipid droplet numbers to marrow area in each measurement region in the Cont and Jump groups are shown (C). The number of lipid droplets in Region I in the Cont and Jump groups was significantly (p < 0.05) smaller than the number in Regions II and III in the same group. The ratios of PPAR $\gamma$ 2-expressing cells to marrow areas in the Cont and Jump groups are shown (D). We performed immunohistochemical staining to detect PPAR $\gamma$ 2 expression in the marrow cells. Data shown are the means  $\pm$  S.D. \*p <

0.05, compared with the Cont group.

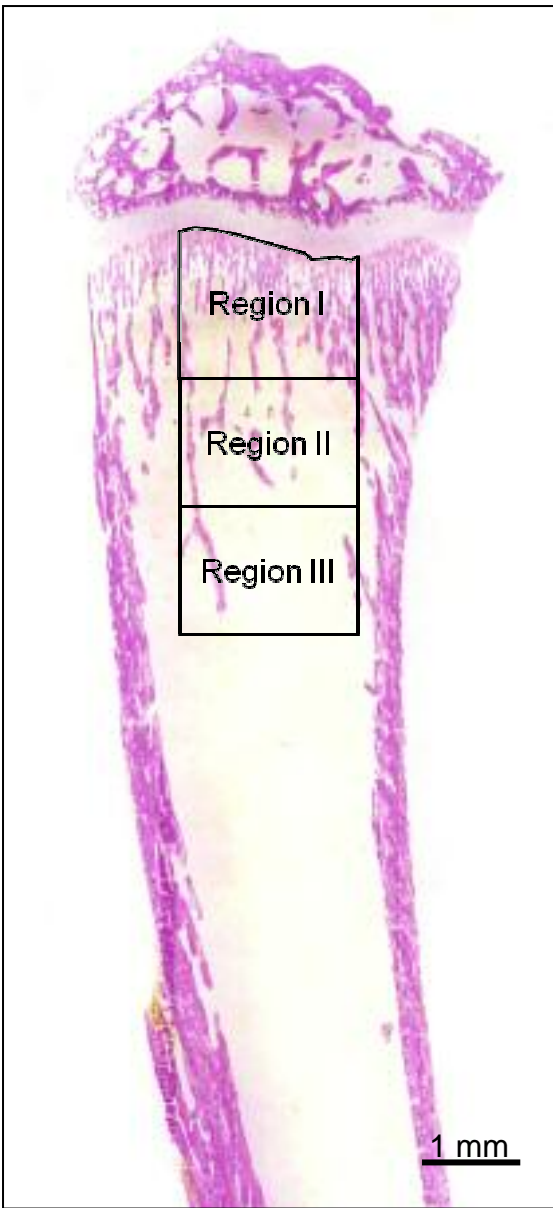
Fig. 5 Expression levels of BMP-2, BMP-4, Runx2, osterix, PPAR $\gamma$ 2, C/EBP $\alpha$ , C/EBP $\beta$ , and C/EBP $\delta$  mRNA in bone marrow cells of the Cont and Jump groups. We synthesized cDNA from RNA extracted from marrow cells. The cDNAs were amplified using real-time reverse transcription-polymerase chain reaction. The amount of target gene expression was normalized to the expression of the  $\beta$ 2 microglobulin gene. Data shown are the means  $\pm$  S.D. \* $p < 0.05$ , compared with the Cont group at the same time point.

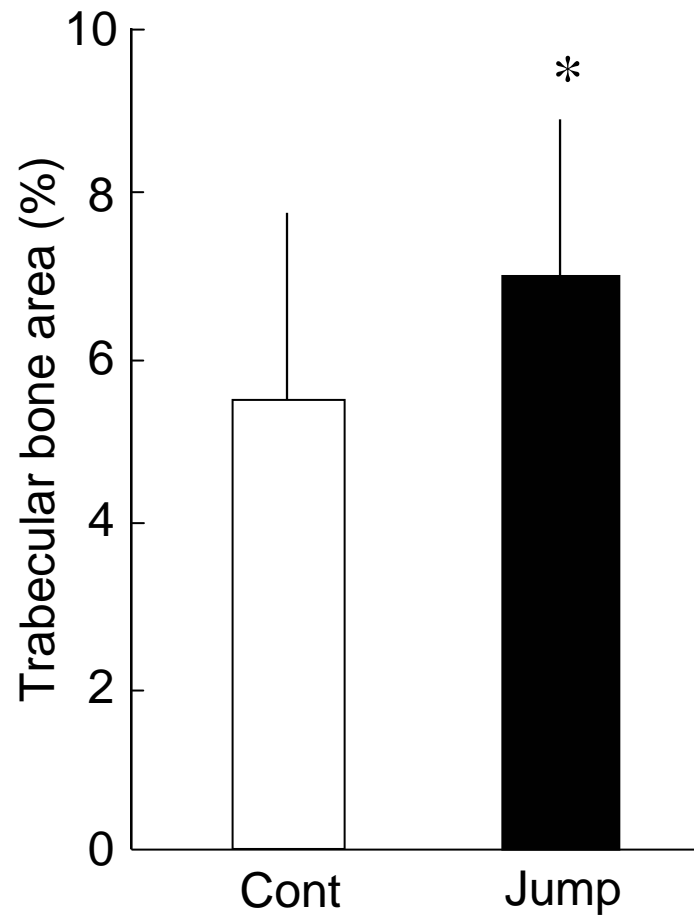
**Table 1**

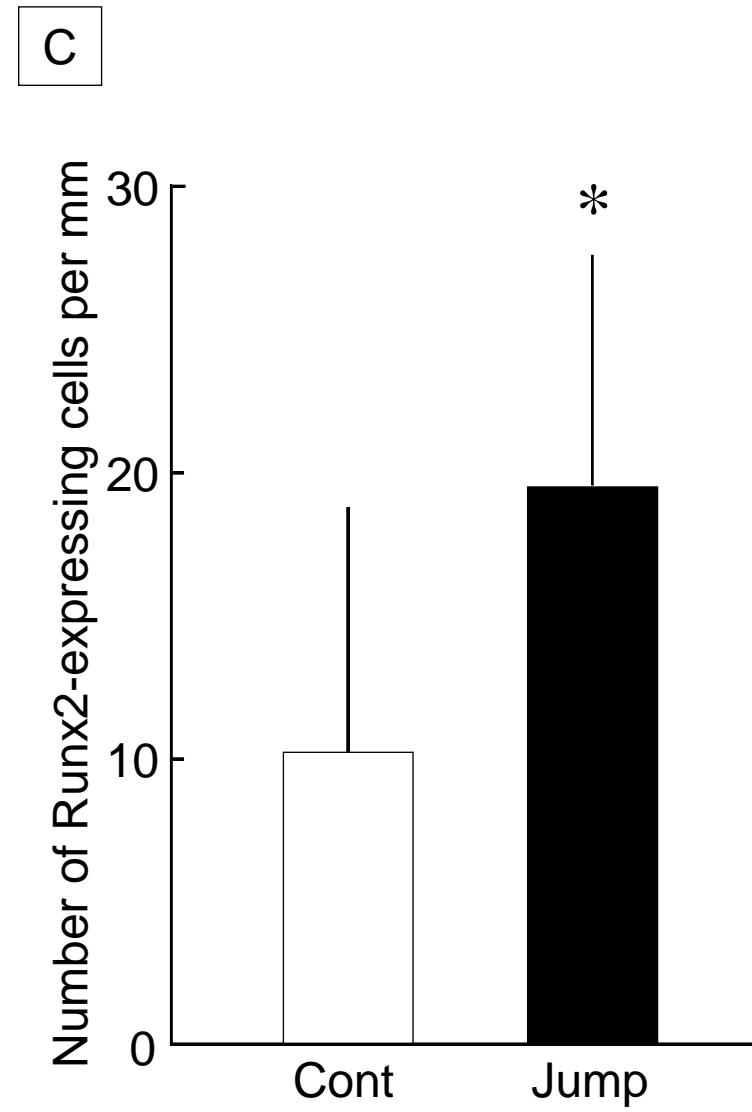
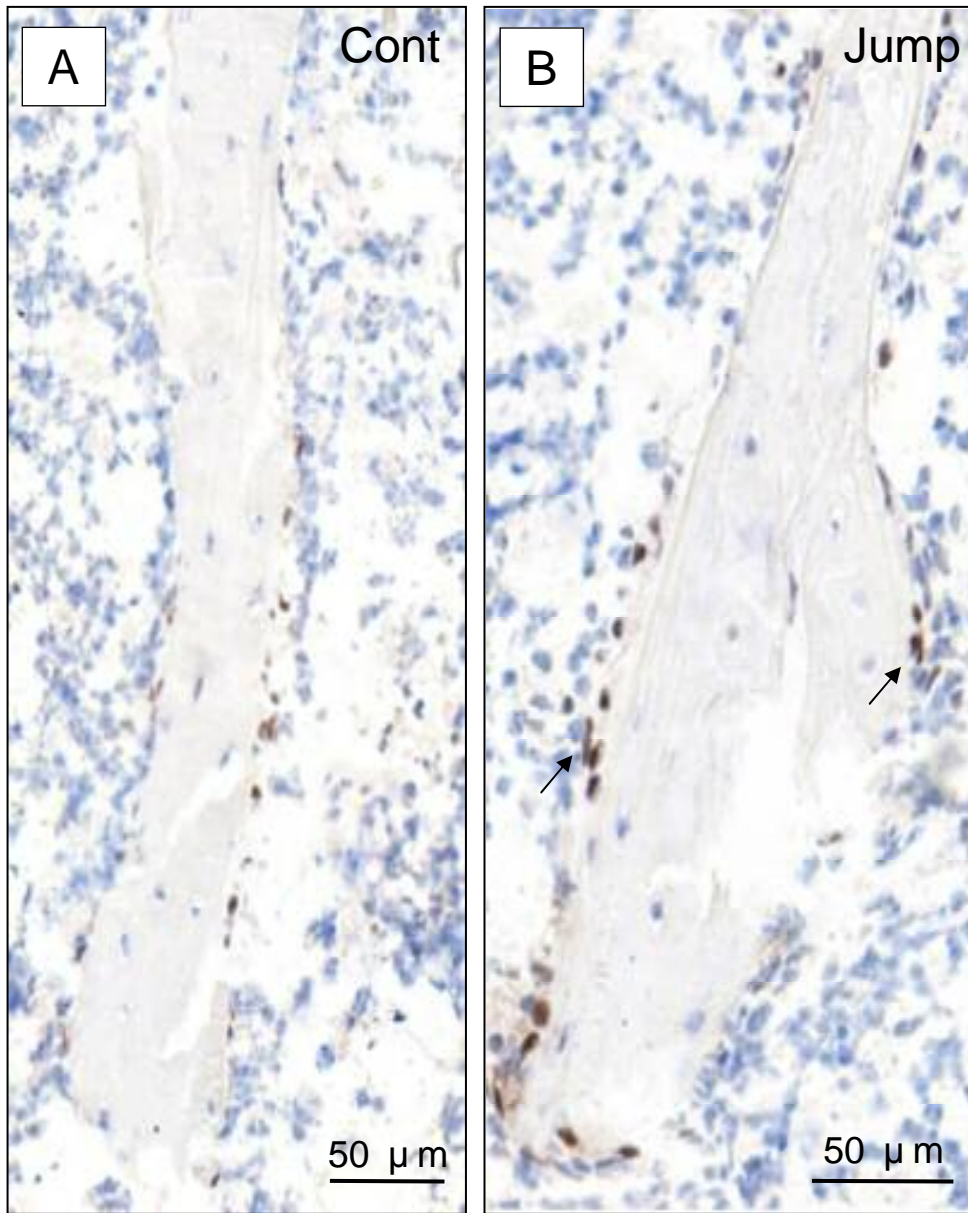
Table 1. Body weight in each group.

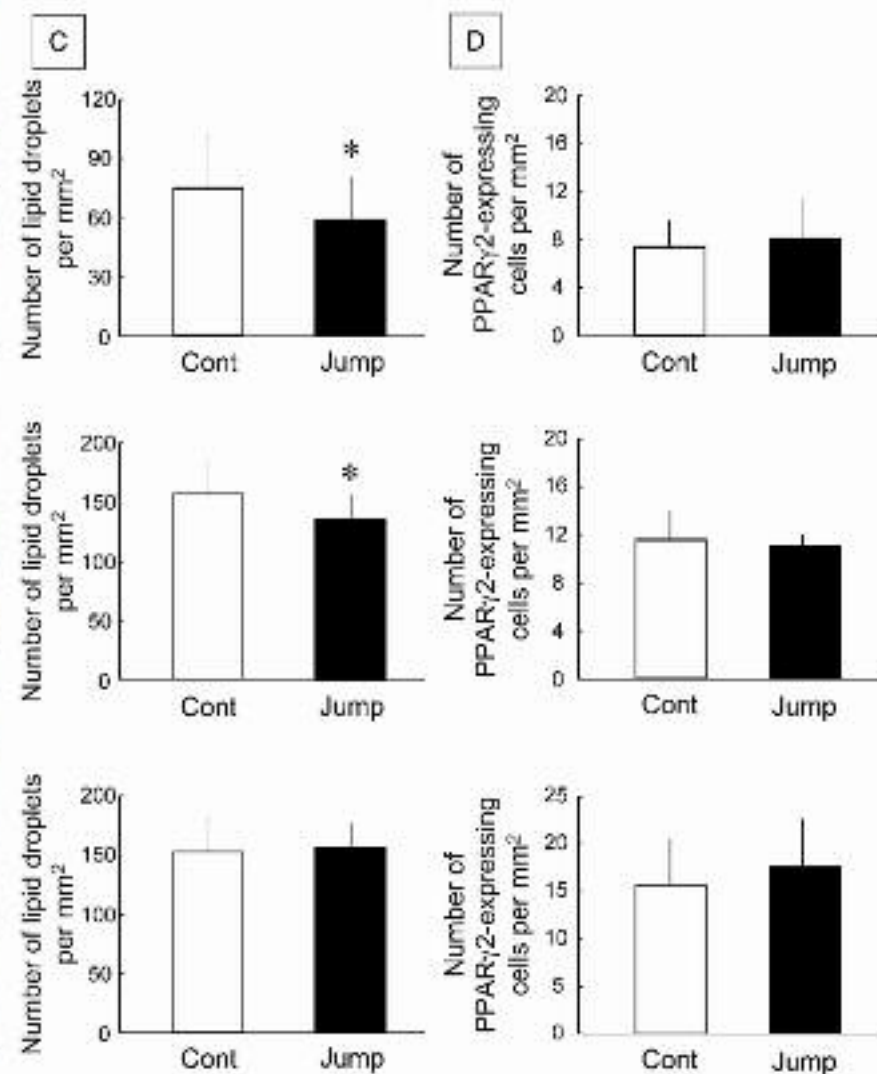
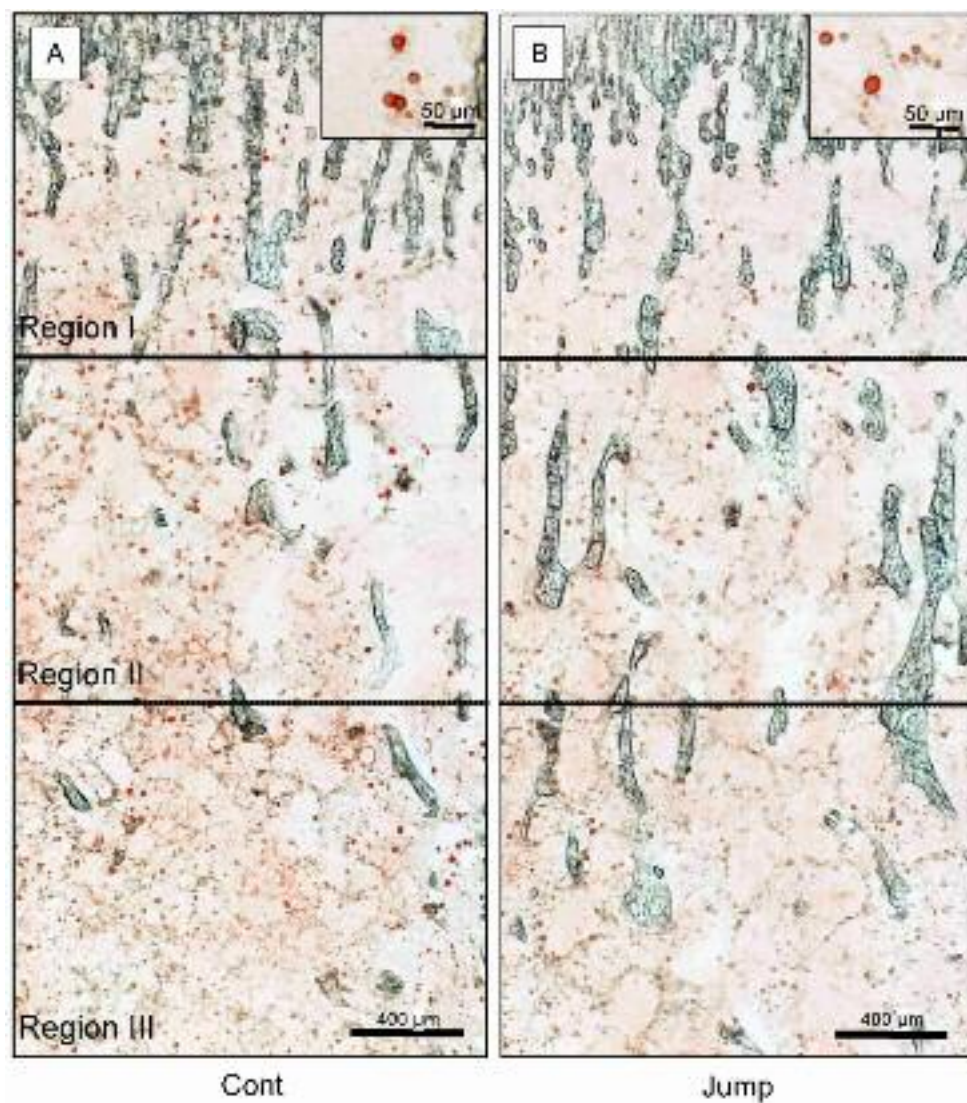
	1 week of training (5 weeks old)		2 weeks of training (6 weeks old)		4 weeks of training (8 weeks old)	
	Cont (n = 5)	Jump (n = 5)	Cont (n = 5)	Jump (n = 5)	Cont (n = 6)	Jump (n = 5)
<i>pre-training</i>	52.2 ± 3.3	53.4 ± 4.6	51.0 ± 2.1	51.8 ± 4.1	50.8 ± 3.7	51.6 ± 4.2
Body Weight (g)						
<i>post-training</i>	65.4 ± 2.3 *	70.6 ± 3.2 *	88.8 ± 2.5 *	91.6 ± 4.2 *	120.2 ± 3.6 *	117.4 ± 4.8 *

Data shown are the means ± S D. \*p &lt; 0.05, compared with the pre-training value in the same group.

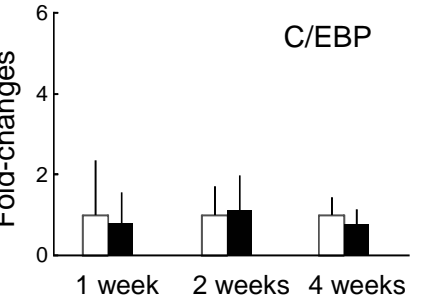
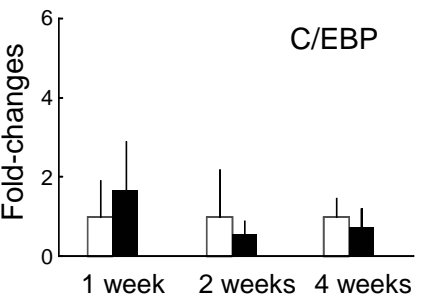
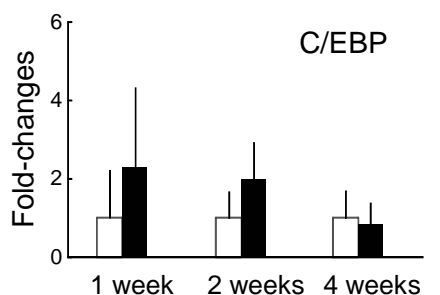
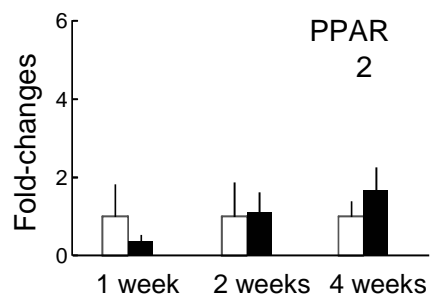
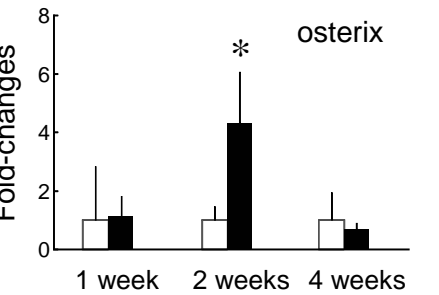
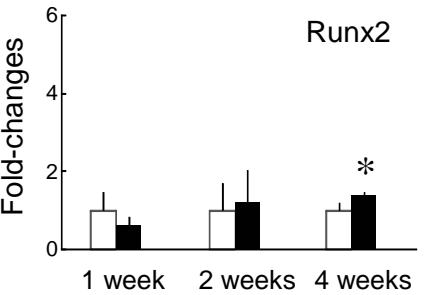
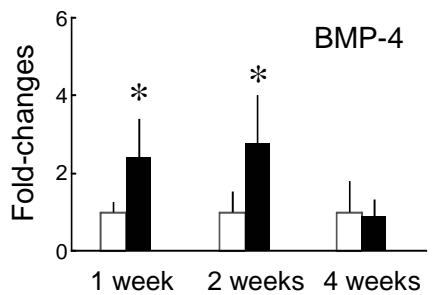
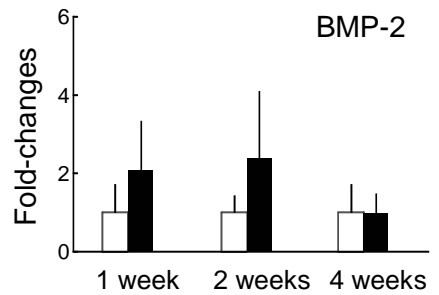












□ Cont      ■ Jump