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Perspective Article

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Changes in trabecular bone turnover and bone marrow cell development in tail-suspended mice

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Abstract

Skeletal unloading induces trabecular bone loss in loaded bones. The tail-suspended mouse model simulates conditions associated with lack of mechanical stress such as space flight for the loaded bones. In such a model, the tail supports the body weight. The forelimbs are normally loaded and the movement of its hindlimbs is free without weight bearing. Histomorphometric analyses of the murine tibiae of the elevated hindlimbs show that trabecular bone volume rapidly diminishes within one week and stabilizes at that level in the subsequent week of tail suspension. Two-week reloading after one-week unloading completely restores trabecular bone volume, but this does not happen after two-week unloading. Unloading for one or two weeks significantly reduces bone formation rate and increases both the osteoclast surface and number compared with age-matched ground control mice. Subsequent reloading restores reduced bone formation and suppresses increased bone resorption. In bone marrow cell cultures, the numbers of alkaline phosphatase (ALP)-positive colony-forming units-fibroblastic (CFU-f) and mineralized nodules are significantly reduced, but the numbers of adherent marrow cells and total CFU-f are unaltered after tail suspension. On the other hand, subsequent reloading increases the number of adherent marrow cells. Unloading for one week significantly increases the number of tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells compared with the control level. Our data demonstrate that tail suspension in mice reduces trabecular bone formation, enhances bone resorption, and is closely associated with the formation of mineralized nodules and TRAP-positive multinucleated cells in bone marrow cultures obtained from tibiae. Two-week reloading restores bone volume reduced after one-week unloading, but does not after two-week unloading. The tail-suspended model provides a unique opportunity to evaluate the physiological and cellular mechanisms of the skeletal response to unloading and reloading.

Keywords: Unloading, Reloading, Trabecular Bone, Bone Marrow, Tail Suspension

Introduction

Skeletal loading plays an important role in the maintenance of bone mass, bone shape and bone strength. Skeletal unloading, such as during space flight and long-term bed rest, induces bone loss in loaded bones in humans[1]. Morey and Baylink[1] reported that bone formation decreased at the periosteum whereas bone resorption remained unchanged during space flight. They also described recovery of suppressed bone formation under normal gravity after space flight. Since then, several studies have shown impaired bone formation or osteoblastic function in various animal models simulating skeletal unloading, such as tenotomy[2], sciatic neurectomy[2-4], elastic bandage fixation[5] and tail suspension[6].

Studies from our laboratory demonstrated that sciatic neurectomy reduced the number of adherent bone marrow cells at 7 and 10 days after immobilization[3]. However, we found no significant difference in this parameter between the tail-suspended mice and ground control mice during the period of tail-suspension[6]. Although a transient increase in bone resorption was observed after neurectomy[3], there was a constitutive increase in bone resorption during the period of tail suspension[6]. Thus, differences in sequential changes in bone formation and resorption in the trabecular bone and bone marrow cells depend on the method of mechanical unloading used.

The purpose of this review is to describe the trabecular bone turnover and bone marrow cell development during mechanical unloading and reloading using tail-suspended mice. We also discuss various physiological responses of bone and bone cells *in vivo* and *in vitro* to mechanical stress.

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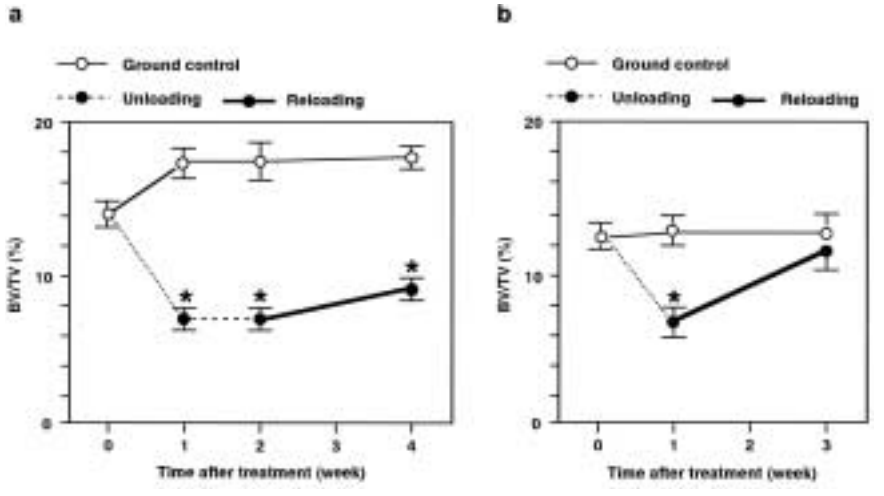

Figure 1. Changes in trabecular bone volume after skeletal unloading and subsequent reloading. (a) Unloading by tail suspension for 2 weeks and subsequent reloading for 2 weeks in 8-week-old ddY male mice. (b) Unloading by tail suspension for 1 week and subsequent reloading for 2 weeks in 8-week-old C57BL/6J male mice. Data are presented as mean±SEM. n = 5-10 mice. BV/TV: percentage of bone volume relative to tissue volume. *p<0.01 vs age-matched ground control mice at each time point by Mann-Whitney U test.

The tail-suspended mouse model

To simulate and investigate in detail certain aspects of space flight, the tail-suspended rat model was first developed at the National Aeronautics and Space Agency (NASA)-Ames Research Center in the mid-1970s[7]. Globus et al.[8] showed a marked bone loss in hindlimbs of growing rats within two weeks of tail suspension compared with their control rats. Indicators of stress, including body weight gain and serum corticosteroid levels, were equivalent to those of the ground control animals[9]. Thus, the tail-suspended rat model is associated with minimal stress to the animals. This model is suitable for research as it can be reloaded easily and recovery occurs in most animals. In addition it complies with the established criteria of animal care. Experimental protocols using hindlimb unloading have been approved by not only the NASA-Ames Animal Care and Use Committee (ACUC), but also numerous other academic research institutions[10].

The tail-suspended mouse model was first reported by Simske and co-workers[11] in 1992. We also used this model and described the effects of mechanical unloading and reloading on trabecular bone turnover and bone marrow cell development[6]. In the mouse model, seven-week-old ddY or C57BL/6J male mice are individually housed in similarly designed cages in an air-conditioned environment (temperature 24±1°C, humidity 55 ± 5%) illuminated from 07:00 to 19:00. During a 1-week period of acclimatization, the mice are fed standard rodent chow (CE-2; Clea Japan Inc., Tokyo, Japan) containing 1.25% of calcium, 1.06% of phosphorus and 2.0 IU/g of vitamin D_3. Control mice are pair fed with tail-suspended mice during the same period. A strip of elastic tape forming half a circle at the center of the tail is applied to the ventral surface of the tail and is secured by another strip of the tape applied spirally to the whole tail. A swivel attached to the half circle of tape is fixed to an overhead wire, the height of which is adjusted to maintain the mice at approximately 30° head down tilt. Thus, in the final position, the tail supports the body weight. The forelimbs are normally loaded and the movement of its hindlimbs is free without weight bearing (unloaded). In this

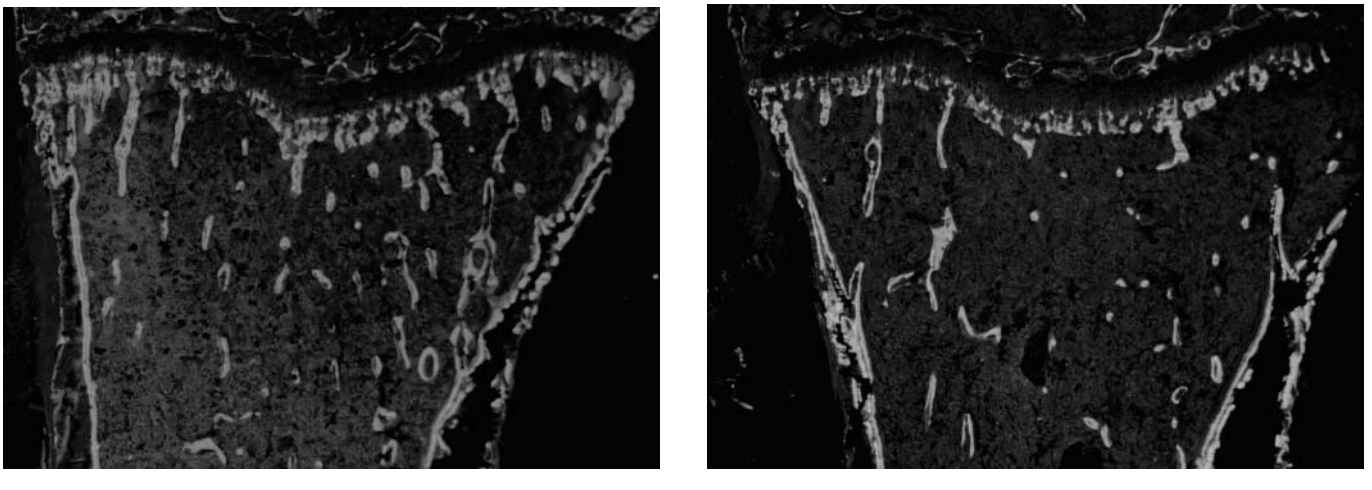

Figure 2. Histological features of the proximal metaphyses of the tibiae from C57BL/6J mice under conditions of both ground control and skeletal unloading by tail suspension. The fluorescence-labeled (mineralizing) trabecular bone surface is shown. Original magnification: x10.

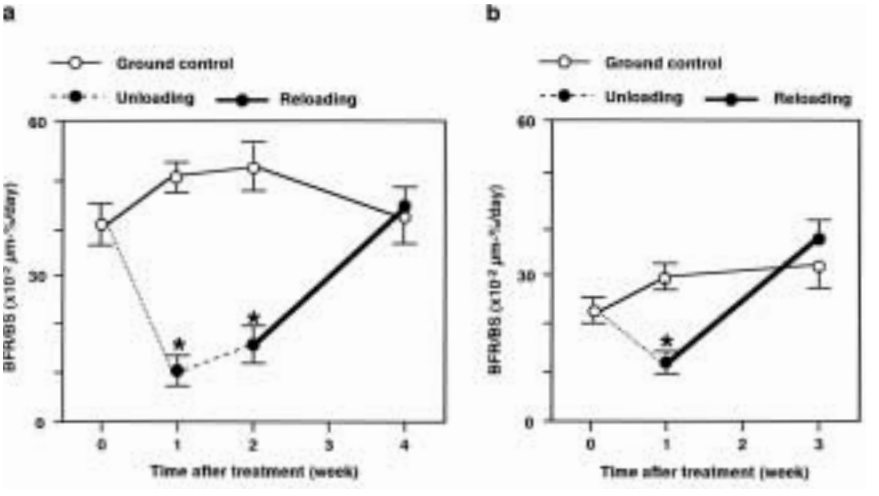

Figure 3. Changes in trabecular bone formation rate after skeletal unloading and subsequent reloading. (a) Unloading by tail suspension for 2 weeks and subsequent reloading for 2 weeks in 8-week-old ddY male mice. (b) Unloading by tail suspension for 1 week and subsequent reloading for 2 weeks in 8-week-old C57BL/6J male mice. Data are presented as mean±SEM. n = 5-10 mice. BFR/BS: percentage bone formation rate relative to bone surface. *$p < 0.01$ vs age-matched ground control mice at each time point by Mann-Whitney U test.

model, the mice are maintained in a suspended position 24 h/day but are allowed free access to food and water by use of their forelimbs during the period of suspension, which ranges from 1-2 weeks, depending on the purpose of the experiment.

Two-week unloading by tail suspension in mice suppresses body weight gain, without a significant difference[6]. The substantial weight loss may suggest stress, unlike the rat model. Knockout mice and transgenic mice are available for the investigation of various genetic functions. We recently reported the baseline histomorphometric and cell culture data[6] for future experiments using knockout and transgenic mice.

Trabecular bone formation and resorption

The length and diameter of the femur gradually increase during the experimental period, and there are no significant differences in both parameters at the end of the study between the tail-suspended and control mice. Histomorphometric analyses of the proximal tibiae of the tail-suspended mice show that the relative trabecular bone volume (BV/TV) rapidly diminishes within the first week of suspension then stabilizes at that level during the subsequent week (Fig. 1). Unloading also significantly reduces mineralized trabecular bone surface (MS/BS) (Fig. 2), mineral apposition rate (MAR) and bone formation rate (BFR/BS) (Fig. 3). This reduction appears to be transient with a marked decrease noted during the first week of suspension. During the second week, the same parameters remain at the same low levels. Unloading significantly increases both the relative osteoclast surface (Oc.S/BS) and number (Oc.N/BS) compared with those in age-matched ground control mice. The substantial body weight loss may account for the increased number of osteoclasts.

Other studies have shown that the periosteal mineralization rate in unloaded cortical bone slows significantly during the first week of hindlimb unloading compared with control in rats[12]. However, the rate of cortical bone mineralization recovers subsequently, although it does not reach control levels as long as the bones are unloaded. Thus, both the number of osteoblasts and bone formation rate are suppressed by unloading, indicating that osteoblasts play a critical role in the skeletal response to unloading. The cellular basis for the recovery of osteoblast number despite continued unloading is not known at present.

Changes in osteoclast number on bone resorption are still controversial, presumably depending on the experimental conditions such as murine strains, age of animals and feeding. Both no change[13,14] and increase[2] in the number of osteoclasts in response to hindlimb unloading have been reported previously in growing rats. Bone resorption at the endosteal surface is not affected by hindlimb unloading.

Bone marrow cell development

Although unloading for 1 or 2 weeks does not alter the number of adherent bone marrow cells per tibia or total colony-forming units-fibroblastic (CFU-f) compared to the control in mice[6], other studies in rats have shown that total CFU-f is reduced after tail suspension[15,16]. This may be a species difference between rats and mice. Unloading for 1 week significantly reduces the number of alkaline phosphatase (ALP)-positive CFU-f and mineralized nodule formation among cultured bone marrow cells of the tibiae compared with the control in mice[6]. Prolonged unloading for 2 weeks results in recovery of these parameters to the control levels.

Unloading for 1 week significantly increases the number of tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells compared with the control in mice[6]. Unloading for 2 weeks tends to increase the number of these cells, albeit insignificantly. The number of colony-forming units for granulocytes and macrophages (CFU-GM) does not change in tail suspended mice or the age-matched controls throughout the unloading period.

The decline in bone formation caused by unloading is mediated by decreases in the rates of proliferation, maturation or activity of osteoblasts and their precursors. Hindlimb unloading causes a reduction in the rate of proliferation of cultured endosteal osteoblasts and marrow stromal cells that express ALP in rats[15]. However, unloading for 2 weeks does not affect ALP activity or the number of cells that synthesize

osteocalcin, osteonectin and collagen in either cell population[15]. Unloading reduces the level of messenger ribonucleic acid (mRNA) for transforming growth factor-β2 (TGF-β2), insulin-like growth factor-2 (IGF-2), osteopontin and osteocalcin in rats[17,18].

Our observations suggest that unloading significantly increases the percentage of hypoploid bone marrow cells, which are considered to reflect predominantly apoptotic cells, relative to control mice. Disruption of p53 gene in response to 1-week unloading does not result in reductions in bone volume and bone formation[19]. We consider that the rapid reduction in bone volume and bone formation after unloading is related to p53 gene-mediated apoptosis of osteoblastic cell lineage.

Reloading

Two weeks of reloading after one week of unloading completely restores trabecular bone volume. However, this effect is not observed following two-week unloading in mice (Fig. 1). In addition, Sessions and colleagues[20] reported that the bone mass and calcium content returned toward, but did not achieve, control levels, when growing rats were unloaded for 2 weeks and then reloaded for 2 weeks. Reloading rapidly accelerates bone formation rate, which returns to control levels by the end of the first week and exceeds control levels the following week (Fig. 3)[6,20]. Stimulation of bone formation rate in bones that were previously unloaded may account for the recovery of bone mass toward control levels.

Recent experiments using the hindlimb unloading model focused on the sequence of molecular events that occur when bone formation is activated during reloading[21,22]. In cancellous bones of rats, TGF-β mRNA levels increase within 8 h of unloading, followed by a rise in mRNA for type I collagen at 12 h, associated with increases in osteocalcin mRNA levels at 16 h. A similar pattern is noted for mRNA purified from whole cortical bone, although the increase in expression levels of these genes does not occur until after 24 h of reloading. Interestingly, mechanical reloading of the hindlimb after 2 weeks of tail suspension in rats causes a transient increase within 2 h in cyclooxygenase (COX)-2 mRNA expression in intraosseous cells, composed mainly of osteocytes, and c-fos expression in periosteal cells[23]. However, because COX-2 expression in osteocytes is not enhanced after 20 min of reloading when c-fos expression is already increased in periosteal cells, the enhancement of c-fos expression does not appear to be mediated by the increased production of prostaglandins (PGs) in osteocytes. Mechanical unloading is associated with impaired periosteal bone formation by reduced expression of c-fos in periosteal cells. It is likely that the intracellular signaling cascade mediates the enhancement of c-fos expression in periosteal cells in response to mechanical stimulation.

Chow[24] tested the ability of nitric oxide (NO) donors, S-nitroso-N-acetyl-D, L-penicillamine (SNAP) and S-nitroso-glutathione (GSNO) to mimic or augment the osteogenic response to a minimal mechanical stimulus. Treatment of rats with SNAP or GSNO resulted in a significant potentiation of this osteogenic response. Inhibition of either PG or NO production at the time of loading caused a partial suppression of c-fos mRNA expression in loaded vertebrae. Both PG and NO are required for mechanical load-induced osteogenesis and they appear to be generated largely independently of each other. Our preliminary studies[25] have shown that inducible NO synthase (iNOS) knockout mice show no increase in trabecular bone volume or bone formation rates in response to reloading after 1-week unloading. In contrast, iNOS wild-type mice respond to reloading with increases in trabecular bone volume and bone formation rate to levels similar to those noted in the age-matched controls. Combined together, these results indicate that generation of NO through iNOS is essential for the stimulation of bone formation upon mechanical loading.

The AP-1 family of transcription factors consists of dimeric complexes of Fos-(c-Fos, FosB, ΔFosB, Fra-1 and Fra-2) and Jun-related (cJun, JunB and JunD) proteins. These proteins participate in the regulation of bone cell proliferation and differentiation[26]. Overexpression of ΔFosB in transgenic mice leads to increased bone formation throughout the skeleton and persistent post-developmental increase in bone mass[27]. In contrast, ΔFosB inhibits adipogenesis both *in vivo* and *in vitro*[27]. Because osteoblasts and adipocytes are thought to share a common precursor, ΔFosB might act on osteoblasto-genesis as a transcriptional regulator, possibly at the expense of adipogenesis, in response to reloading.

Signal transduction mechanism

Mechanotransduction in bone cells is poorly understood, and is thought to be influenced by several modulators (Fig. 4)[28]. Various *in vitro* experiments have been performed using shear stress produced by fluid flow and stretching. Cellular responses are considered to employ at least two different intracellular signal transduction pathways[28].

The first pathway is a mechanotransducer that resides in the cell membrane and is stimulated through seven transmembrane hormone receptors. This transducer is guanidine nucleotide-binding protein (G protein)-linked and also interacts with a stretch-activated cation channel. The second pathway involves a direct linkage between trans-membrane integrins and the actin cytoskeleton. Induction of MAP kinase and cFos/cJun is dependent on this pathway. Stimulation of PGs can be blocked by 70-80% within G protein inhibitors GDPβS and pertussis toxin by 83% and 72% respectively, *in vitro*[29].

The most likely intermediaries for cell-to-cell communication between sensor cells and effector cells are PGs and NO. Mechanical stimuli applied to cell and tissue culture induce production of several PGs and NO. Interestingly, blockage of PG production *in vivo*, particularly with selective inhibitors of the inducible COX-2, eliminates mechanically-induced bone formation[30,31]. Inhibitors of NO synthase also suppress mechanically-induced bone formation in rats[32,33].

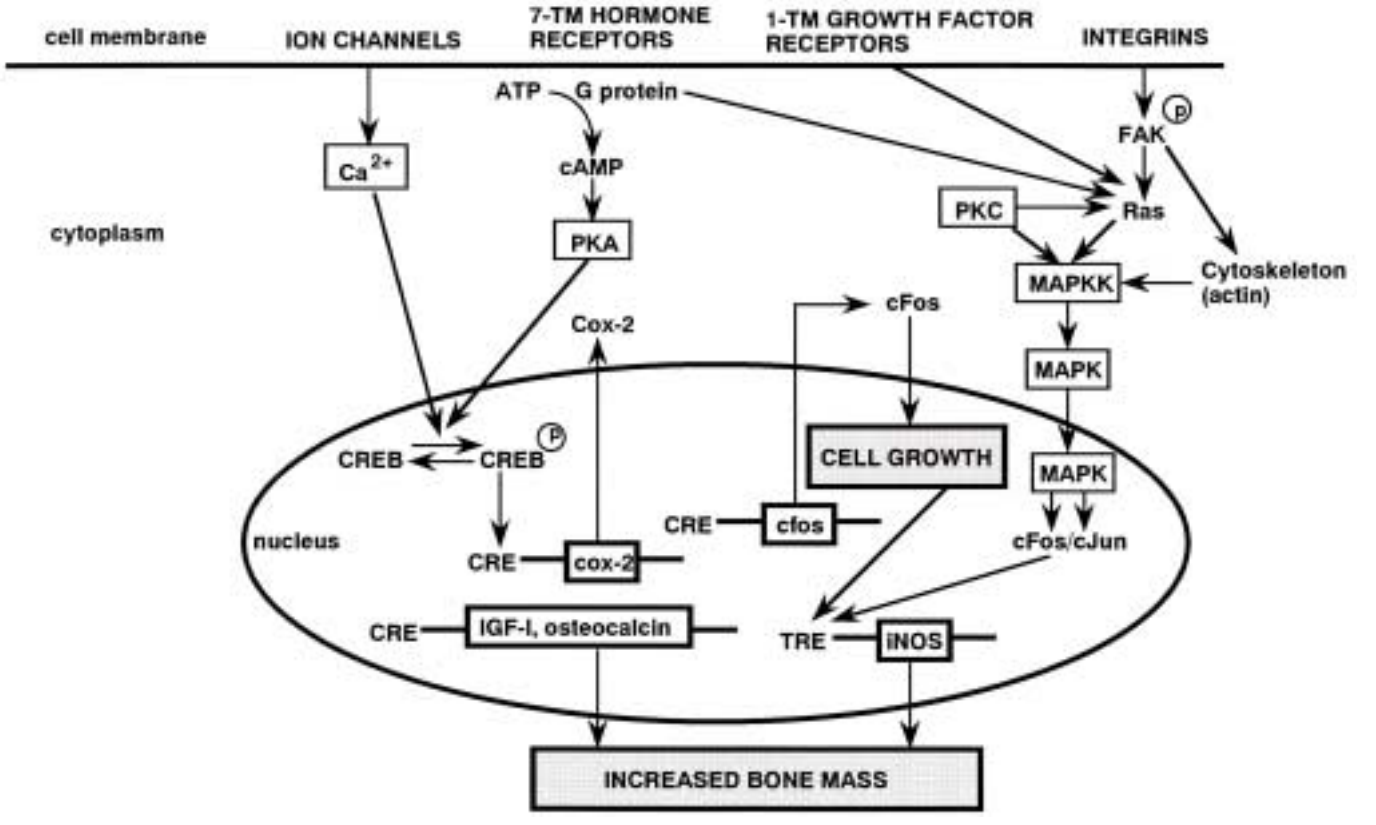

Figure 4. *In vitro* signal transduction pathways in mechanically-stimulated osteocytes and osteoblasts. TM, transmembrane; G protein, guanidine nucleotide-binding protein; FAK, focal adhesion kinase; PKA, protein kinase A; PKC, protein kinase C; MAPK, MAP kinase; COX, cyclooxygenase; CRE(B), cyclic AMP-responsive element (binding protein); TRE, tyrosine kinase-responsive element; iNOS, inducible nitric oxide synthase.

Conclusion

The murine tibiae of the elevated hindlimbs show a rapid reduction of trabecular bone volume within one week, which persists during the subsequent week. Two-week reloading following one-week unloading completely restores trabecular bone volume, but this does not happen after two-week unloading. Trabecular bone formation is reduced and bone resorption is enhanced after tail suspension in mice, closely associated with the formation of mineralized nodules and TRAP-positive multinucleated cells in the marrow cultures obtained from tibiae. In conclusion, hindlimb unloading is a useful model for investigating the cellular mechanisms that mediate the skeletal responses to bone unloading and reloading.

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