The Central Nervous System (CNS)-independent Anti-bone-resorptive Activity of Muscle Contraction and the Underlying Molecular and Cellular Signatures

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Background: Mechanisms by which muscle regulates bone are poorly understood.

Results: Electrically stimulated muscle contraction reversed elevations in bone resorption and increased Wnt signaling in bone-derived cells after spinal cord transection.

Conclusion: Muscle contraction reduced resorption of unloaded bone independently of the CNS, through mechanical effects and, potentially, nonmechanical signals (e.g. myokines).

Significance: The study provides new insights regarding muscle-bone interactions.

Muscle and bone work as a functional unit. Cellular and molecular mechanisms underlying effects of muscle activity on bone mass are largely unknown. Spinal cord injury (SCI) causes muscle paralysis and extensive sublesional bone loss and disrupts neural connections between the central nervous system (CNS) and bone. Muscle contraction elicited by electrical stimulation (ES) of nerves partially protects against SCI-related bone loss. Thus, application of ES after SCI provides an opportunity to study the effects of muscle activity on bone and roles of the CNS in this interaction, as well as the underlying mechanisms. Using a rat model of SCI, the effects on bone of ES-induced muscle contraction were characterized. The SCI-mediated increase in serum C-terminal telopeptide of type I collagen (CTX) was completely reversed by ES. In ex vivo bone marrow cell cultures, SCI increased the number of osteoclasts and their expression of mRNA for several osteoclast differentiation markers, whereas ES significantly reduced these changes; SCI decreased osteoclast numbers, but increased expression in these cells of receptor activator of NF-κB ligand (RANKL) mRNA, whereas ES increased expression of osteoprotegerin (OPG) and the OPG/RANKL ratio. A microarray analysis revealed that ES partially reversed SCI-induced alterations in expression of genes involved in signaling through Wnt, FSH, parathyroid hormone (PTH), oxytocin, and calcineurin/nuclear factor of activated T-cells (NFAT) pathways. ES mitigated SCI-mediated increases in mRNA levels for the Wnt inhibitors DKK1, sFRP2, and sclerostin in ex vivo cultured osteoblasts. Our results demonstrate an anti-bone-resorptive activity of muscle contraction by ES that develops rapidly and is independent of the CNS. The pathways involved, particularly Wnt signaling, suggest future strategies to minimize bone loss after immobilization.

Muscle and bone are intimately connected both functionally and physically. Muscle contractions cause the largest physiological loads on bone, and there is a linear relationship between muscle size or force and bone mineral content and bone strength in healthy adults (1, 2). Although muscle contraction or the absence thereof plays a clear role in the interaction of these tissues, the potential cellular and molecular mechanisms by which muscle activity is coupled to bone mass are poorly understood.

Spinal cord injury (SCI) is a life-long and life-changing injury that results in severe immobilization, paralysis, muscle atrophy, loss of strength, and rapid, severe sublesional bone loss (3–5).
After SCI, bone loss proceeds at rates as great as 1% of bone per week for the first year after injury with the greatest bone loss observed at the distal femur and proximal tibia (4). When the spinal cord has been severed completely, all neural connections between the central nervous system (CNS) and bone are disrupted, which distinguishes SCI from other conditions of immobilization. Bone is richly innervated by autonomic and sensory nerve fibers, which have been implicated in regulating bone mass (5). Remobilization at least partially restores bone mass in animal models of hindlimb suspension (6–8) or astronauts returning from space (9), although the cellular and molecular mechanisms that underlie this anabolic bone response are not well understood. Interestingly, muscle contraction elicited by electrical stimulation (ES) partially protects against SCI-related bone loss and may even partially reverse it (3–5). Therefore, the application of ES after SCI provides a unique opportunity to study the effects of muscle contractions on bone biology and roles of the CNS in this interaction, e.g. the roles of the CNS when bone is reloaded through stimulation of muscle contractions, as well as the cellular and molecular events by which osseous responses to reloading occur.

Canonical Wnt signaling involves binding of a Wnt to Frizzled receptors leading to nuclear translocation of β-catenin and transcriptional regulation, which, to a large degree, occurs through binding of β-catenin to TCF/LEF family transcription factors (10, 11). There is now considerable evidence that the Wnt/β-catenin pathway is a key determinant of bone mass and that regulation of this pathway in bone by Wnt inhibitors contributes to bone loss (10). Rapid and extensive declines in bone mass in the conditions of unloading of bone by spaceflight or immobilization such as SCI are mainly attributed to increased bone resorption and diminished bone formation (4). The pathogenesis of bone loss due to unloading is believed to include inhibition of canonical Wnt signaling through the release from osteocytes of sclerostin and resultant increase in osteocyte release of RANKL (receptor activator of NF-κB ligand (12–14)); RANKL recruits hematopoietic cells of the monocyte lineage to osteoclastic differentiation and stimulates bone resorption by mature osteoclasts (15). A host of other inhibitors of canonical Wnt signaling is known, such as DKK1 and sFRPs (soluble frizzled-related proteins), although their role in bone loss of immobilization is unclear (11). Sclerostin expression is inversely related to bone loading (16) and must be reduced for anabolic responses to bone loading (17). Conversely, canonical Wnt signaling stimulates release by osteoblasts of osteoprotegerin (OPG) (18), an inhibitor of RANKL (15).

The goals of the current study were to determine how bone responds to reloading through muscle contraction after a prolonged period of unloading achieved by completely transecting the spinal cord that resulted in complete disconnection of the brain from bone. The effects of reloading of bone elicited by ES-stimulated muscle contraction for 7 days when initiated at 16 weeks after SCI were studied. Effects of ES were examined on bone metabolism, differentiation of bone marrow progenitor cells, molecular signaling pathways represented by differentially expressed genes, and targeted and global gene expression analysis of differentiated osteoblasts and osteoclasts obtained by culturing bone marrow progenitors was performed.

**EXPERIMENTAL PROCEDURES**

**Animals**—An animal model was developed wherein contraction of muscles of the hindlimb was stimulated 16 weeks after SCI by use of single channel, programmable implantable microstimulators. In this model, ES elicited near isometric contractions of ankle flexors and extensors. All animal studies were approved by the Institutional Animal Care and Use Committee at the James J. Peters Veterans Affairs Medical Center and conformed to all guidelines and regulations for the protection of the welfare of animal subjects. Female Wistar rats 8 weeks of age were obtained from Taconic Farms (Hudson, NY), housed in a temperature- and humidity-controlled room providing a 12:12-h day:night cycle, and fed food and water ad libitum.

**Experimental Design**—Three groups of animals were studied: spinal cord transected (SCI animals), spinal cord- transected with implantable ES devices (SCI-ES animals), and a sham-SCI group (Sham animals). SCI animals and SCI-ES animals underwent complete transection of the spinal cord at the interspace between the 9th and 10th thoracic vertebrae as described previously (19). Sham animals underwent an identical surgery except that the spinal cord was not cut. At 14 weeks after SCI, a microstimulator was implanted into SCI-ES animals with electrodes placed adjacent to the anterior tibial nerve and close to the common peroneal nerve, as described in a separate study. The distal insertion of the gastrocnemius muscle was cut at this time. SCI and Sham animals underwent an identical procedure except that no stimulator was implanted; the procedure was carried out at 11 weeks after the sham-SCI surgery for the Sham group.

The ES was begun during the 16th week following spinal cord transection and elicited near isometric contraction of soleus and plantaris opposed by contraction of the tibialis anterior. Stimulation was provided for 60 min on each training day and consisted of brief periods of contraction (2 s) at 40 Hz and 1.5 V and 18 s of rest. The temporal sequence of surgeries and ES is summarized in Fig. 1A.

Collection of blood and tissues from SCI animals and SCI-ES animals occurred at 17 weeks after SCI or 8 days after initiating ES. Tissues were collected from Sham animals at ~14 weeks after surgery. Animals were anesthetized by inhalation of isoflurane followed by removal of the plantarous muscles after careful dissection and collection of blood by ventricular puncture. Animals were euthanized by aortic transection, and tibia and femur were removed as a single piece, leaving the knee joint intact, and placed in α-MEM. Muscles were weighed; weights were normalized to body weight before surgeries to control for individual variations in size.

**Bone Densitometry**—Areal BMD measurements were performed by using a small animal bone densitometer (Lunar PIXImus, Inside Out Sales, Fitchburg, WI). The femur and tibia were placed on a tray with the knee forming a 135° angle. Distal femur and proximal tibia were selected as regions of interest.

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5 Y. Wu, L. Collier, W. Qin, G. Creasey, W. A. Bauman, J. Jarvis, and C. Cardozo, submitted for publication.
The instrument was calibrated using a phantom per the manufacturer’s recommendation. The coefficient of variation for BMD measurements is ~1.5% for the regions of interest.

**Micro-computed Tomography Analysis of Trabecular Architecture**—The distal femur was imaged using a Scanco μCT scanner as described in greater detail in the supplemental Methods. Bone morphometry nomenclature followed established standards (20).

**Serum Levels of CTX and Osteocalcin**—Blood samples were centrifuged at 2,000 rpm for 10 min at 4 °C, and the serum was separated and then stored at −80 °C. Serum C-terminal telopeptide of type I collagen (CTX) levels were measured using a RatLaps™ enzyme-immunoassay kit from Immunodiagnostic Systems (Fountain Hills, AZ). Serum concentrations of osteocalcin were measured using a rat osteocalcin enzyme-linked immunoassay kit from Biomedical Technologies Inc. (Stoughton, MA). All samples were assayed in duplicate, following the manufacturer’s recommended procedures.

**Ex Vivo Osteoclastogenesis and Osteoblastogenesis Assays**—Procedures were adapted from those we have previously described (21, 22). Briefly, bone marrow cells were flushed from femora and tibiae of rats with α-MEM, pelleted, and resuspended in this medium and then seeded into wells using an equal number of cells in each well. For *ex vivo* culture of osteoclasts, cells were cultured for 2 days in α-MEM supplemented with human macrophage colony-stimulating factor (M-CSF) (5 ng/ml) after which nonadherent cells were collected and purified by Ficoll-Plus (Amersham Biosciences) and then seeded into wells, again with an equal number of cells per plate, and cultured in α-MEM containing M-CSF (30 ng/ml) and RANKL (60 ng/ml) for 5 days. Cells were then stained for TRAP using a kit (Sigma-Aldrich). The number of TRAP-positive multinucleated cells was then counted; a parallel set of osteoclast cultures at day 5 was used to extract total RNA for microarray analysis as described below.

For *ex vivo* culture of osteoblasts, bone marrow cells were cultured in α-MEM supplemented with 15% preselected FCS (HyClone, Logan, UT) and ascorbic acid-2-phosphate (1 mm). At 10 days, the recruitment of mesenchymal cell progenitors into the osteoblastic lineage was assessed by counting the number of alkaline phosphatase-positive colonies (CFU-f) (Sigma); a parallel set of osteoblast cultures at day 10 was used to extract total RNA for microarray analysis as described below. In separate plates, culture was continued until day 28, when the number of colonies producing mineralized bone matrix (CFU-ob) was determined after von Kossa staining.

**Microarray Analysis**—Microarray analysis was performed using total RNA from *ex vivo* cultured osteoblasts and osteoclasts from the Sham, SCI, and SCI-ES groups. Total RNA was extracted from *ex vivo* cultured osteoblasts and osteoclasts using phenol-chloroform (23–25) and then further enriched using RNeasy minicolumns (Qiagen, Valencia, CA). Microarray analysis was performed by the Microarray Core Facility at the Children’s National Medical Center using rat Illumina mRNA bead profiling arrays. Each sample was analyzed using three separate arrays, and values for replicate arrays were averaged. Expression values were exported from GenomeStudio after base-line subtraction and then processed using the Lumi pack-

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The statistical significance of differences among means was tested using one-way analysis of variance and a Newman–Keuls test to determine the significance of differences between individual pairs of means using a *p* value of 0.05 as the cutoff for significance. Statistical calculations were performed using Prism 4.0c (GraphPad Software, La Jolla, CA).

**RESULTS**

**Effect of Short Term Muscle Contraction on Muscle Weight and Bone Mass**—Weights of plantaris muscle in SCI animals were reduced to 54% of those for Sham animals (Fig. 1B). The weight of plantaris muscle from the ES-exercised left hindlimb was increased significantly by about 35% by ES when compared with the unexercised right hindlimb (Fig. 1B). The mRNA levels of two muscle-restricted ubiquitin E3 ligases linked to muscle atrophy, *Mafb* and *Murf1*, were up-regulated in the plantaris in SCI rats and down-regulated by 7 days of ES (supplemental Fig. 1). Thus, ES stimulated sufficient contractile force to initiate muscle hypertrophy. By inference, ES also restored mechanical loading to bone.

When assessed by micro-computed tomography analysis of trabecular bone of the distal femur, bone volume over total volume (BV/TV), trabecular number (Tb.N), and trabecular thickness (Tb.Th) were reduced and trabecular spacing (Tb.Sp) was increased at 17 weeks after SCI (Fig. 1C). Areal BMD was significantly reduced at 17 weeks after SCI by ~17% for distal femur and 12% for proximal tibia (Fig. 1D). As expected, after only 1 week of reloading of bone, ES did not result in any measurable change in BMD or trabecular number, spacing, or thickness (Fig. 1).

**Muscle Contraction by ES Reduced Markers of Bone Resorption and Osteoclastogenesis**—Serum CTX levels were elevated by ~2-fold in SCI animals (Fig. 2A). One week of ES reduced serum levels of CTX to levels that were similar to those of Sham animals (Fig. 2A). *Ex vivo* cultures of bone marrow hematopoietic stem cells for the SCI group contained significantly more TRAP⁺ multinucleated cells than cultures for the Sham group (Fig. 2, B and C). One week of ES reduced the number of TRAP⁺ multinucleated cells for the SCI group compared with the Sham group (Fig. 2, B and C).
multinucleated cells in the SCI-ES group when compared with the SCI group by 22%, although numbers of cells remained greater than the Sham group (Fig. 2, B and C).

Serum osteocalcin levels were suppressed by slightly more than 50% in SCI animals and were not altered by 1 week of ES (Fig. 3 A). There was a reduction of ∼25% in CFU-ob for the SCI group when compared with the Sham group (Fig. 3, C and D). A similar reduction in CFU-f was observed after SCI (Fig. 3, B and D). ES did not alter CFU-f or CFU-ob (Fig. 3, B–D).

**Gene Expression in ex Vivo Cultured Osteoblasts**—In ex vivo cultures of osteoblasts derived from bone marrow stromal stem cells, mRNA levels for the differentiation markers Runx2, osteocalcin, and bone sialoprotein (BSP) were reduced by ∼70% for each transcript in the SCI group when compared with the Sham group (Fig. 4 A). Levels of these transcripts were not significantly altered by ES (Fig. 4 A). Levels of mRNA for OPG were reduced by ∼40% for SCI rats when compared with Sham rats (Fig. 4 A), associated with 5-fold elevations in mRNA levels for RANKL (Fig. 4 A). One week of ES increased OPG mRNA levels to those found in Sham rats but did not significantly alter mRNA levels for RANKL (Fig. 4 A). Expression of SOST and DKK1 was increased by more than 10-fold for SCI rats when compared with Sham rats; sFRP2 expression was increased 3-fold in the SCI group when compared with the Sham group (Fig. 4 A). ES reduced the expression of DKK1 and sFRP2 to values observed in the Sham group and reduced SOST expression by ∼25% (Fig. 4 A). Expression of Wnt3a and Wnt5a was modestly (3–4-fold) increased in the SCI group when compared with the Sham group; ES greatly increased Wnt3a expression but did not alter Wnt5a mRNA levels (Fig. 4 A). Expression of the Wnt/β-catenin-responsive gene Enc1 (29) was reduced for the SCI group when compared with the Sham group, but was increased for the SCI-ES group when compared with the Sham group (Fig. 4 A).
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The expression of Pthr1 was increased more than 4-fold for the SCI group when compared with the Sham group; ES reduced Pthr1 expression levels observed for the Sham group (Fig. 4A). The expression of genes involved in calcineurin/NFAT signaling, Mef2d (myocyte-specific enhancer factor 2D) (30), and in osteoclastogenesis, Smad3 (31), was also examined. For the SCI group when compared with the Sham group, Mef2d expression was reduced by approximately one-third, but Smad3 expression was unaffected (Fig. 4A). For the SCI-ES group when compared with the SCI group, Mef2d levels were increased to values similar to those in the Sham group, but Smad3 expression was reduced by ~75% (Fig. 4A). Expression of c-fos, a gene up-regulated by PTH in osteoblasts (32), was increased after SCI by ~15-fold with the level reduced, albeit not significantly, by ES (Fig. 4A).

To identify signaling pathways regulated by unloading of bone after SCI and/or reloading of bone by ES, a pathways analysis of differentially expressed genes identified by microarray analysis was performed (supplemental Table 1). Among the enriched pathways identified were Hedgehog-PTH, Wnt, calcium/NFAT, and β-adrenergic signaling (Fig. 4B).

Gene Expression in ex Vivo Osteoclast Cultures—In ex vivo cultures of osteoclasts, levels for transcripts encoding the calcitonin receptor (CTR), integrin β3, and TRAP were all increased by nearly 2-fold for each transcript in the SCI group when compared with the Sham group (Fig. 5A). Expression of Smad3 and c-fos, which have been linked to osteoclastogenesis (31, 33), was also increased by SCI (Fig. 5A). Levels of these mRNAs were reduced by ES (Fig. 5A), with levels for calcitonin receptor, Smad3, and integrin β3 reduced to levels similar to or below those for Sham animals (Fig. 5A). Expression of Pthr1 was reduced by ~60% in the SCI group when compared with the Sham group, with levels being unaltered in SCI animals by ES (Fig. 5A). A pathways analysis of differentially regulated genes identified by microarray analysis (supplemental Table 2) revealed that enriched pathways included signaling through PTH, vitamin D, oxytocin, FSH, and calcium/NFAT (Fig. 5B).

DISCUSSION

Our studies utilized a novel animal model in which the effects of bone reloading due to ES-induced neuromuscular stimulation resulting in muscle contractions were studied 17 weeks after immobilization and extreme unloading of sublesional bone in the absence of neural inputs by higher centers of the CNS due to SCI. The major effects of ES included reductions in serum levels of a biochemical marker of bone resorption (CTX) and of the number of bone marrow hematopoietic progenitors forming osteoclasts when cultured ex vivo. A biochemical marker of bone formation, osteocalcin, was reduced by SCI but not increased by 7 days of ES in SCI animals. Although the number of osteoblast progenitors was reduced by SCI and unaltered by 7 days of ES, there were nonetheless several important and potentially beneficial changes in gene expression in the osteoblasts derived from these cells; ES altered gene expression for signaling pathways responsible for osteoblast differentiation and function, as well as for regulation of osteoclasts by cells of the osteoblast lineage. Most important among these were Wnt signaling molecules and RANKL and OPG. Thus, our data demonstrate for the first time an anti-bone-resorptive activity of muscle contraction elicited by ES and provide the first evidence about global cellular and molecular alterations in bone after muscle contraction.

Reloading Bone through Muscle Contractions Elicited by ES in a Rat Model of Extreme Bone Unloading by Complete Spinal Cord Transection—The ES protocol and electrode placement employed in the present study produced near isometric contraction of selected ankle extensors (soleus and plantaris) and a major ankle flexor, the tibialis anterior. Seven days of ES down-regulated the expression of the muscle atrophy-related genes Mafbx and MurF1, indicating the occurrence of initial adaptations of skeletal muscle to resumption of neuromuscular activity, thereby confirming that ES produced physiologically significant neuromuscular stimulation. Co-contraction of flexors and extensors would be expected to produce a primarily compressive load on the tibia. This experimental approach provides a unique model to study the effects of muscle and bone interactions and underlying molecular and cellular mechanisms as
they relate to osseous responses to reloading of bone after immobilization.

Osseous effects of ES-based muscle contraction might not be due only to mechanical loading of bone. Biochemical signals emanating from muscle may also play important roles in the effects of muscle contraction by ES on bone. Muscle and bone are intimately associated endocrine organs (34, 35). A growing body of evidence indicates that endocrine and paracrine signals derived from muscle (referred to as myokines) modulate bone metabolism (36). Myokines may include IGF-1, IL-6, leukemia inhibitory factor, fibroblast growth factor 21, myostatin, and follistatin-like 1 (36). Moreover, muscle cells naturally secrete factor(s) that preserve osteocyte viability through the activation of β-catenin (37). It should be appreciated that following SCI, muscle is paralyzed, and that there is little neuromuscular stimulation of muscle innervated by motor neurons originating in spinal segments below the level of the spinal cord injury. Following stroke, paralyzed muscle atrophies and expresses elevated levels of cytokines such as TNF-α and, potentially, myostatin, and increased neuromuscular activity attenuates these abnormalities (38, 39). It may well be that similar abnormalities are present in paralyzed skeletal muscle after SCI; in support of this possibility, preliminary studies have indicated that in male rats, mRNA levels for TNF-α and IL-6 are elevated in gastrocnemius muscle after SCI.6 It might be anticipated that future studies will define the potential roles of soluble mediators originating in skeletal muscle that are responsible for humoral muscle-bone interactions in response to muscle contraction by ES.

Effects of Reloading by ES on Bone Mass, Metabolism, and Differentiation of Bone Marrow Progenitor Cells—Studies of reloading in humans suggest that bone is capable of some increases in mass even after prolonged unloading and extensive loss of bone mass. When used for resistance training, ES pro-

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6 Y. Wu and C. Cardozo, unpublished observations.
motes gains in bone, muscle mass, and strength in persons with SCI (3–5), and a gradual increase in bone has been observed over the first year after return to earth from prolonged spaceflight (9). Our data demonstrate that even after a relatively prolonged period of extreme hindlimb unloading due to SCI, relatively brief periods of reloading for 7 days by ES were sufficient to normalize serum CTX and osteoclast-forming activity of bone marrow hematopoietic precursors and favorably alter gene expression in osteoblasts derived from bone marrow stromal cells. Thus, even after a fairly long period of extreme immobilization, bone is capable of rapidly responding to even a modest increase in mechanical loading. In our studies, we found no effect of ES on bone mass, which is consistent with prior work in adult rats. Approximately 24 weeks of reloading of hind limbs in adult rats was required to restore bone mass to normal, suggesting that rebuilding of bone in adult animals is a rather slow process. By contrast, in a study of young tail-suspended rats for brief periods of 2 weeks, 6 days of reloading normalized depressed bone formation rates (40); in a separate although similar study, 2 weeks of reloading restored normal bone mass and increased bone formation rate and osteoblast numbers (41).

Very little is known about the cellular and molecular mechanisms by which the mass of bone increases after reloading. In rats, reduced bone formation rates were associated with diminished ability of marrow stromal cells to proliferate and undergo osteoblastogenic differentiation after as little as 2 weeks of tail suspension; these abnormalities were normalized by reloading (41, 42). Findings in our study that the potential of bone marrow stromal cells to differentiate into bone nodule-forming osteoblasts was reduced at 17 weeks after SCI suggest that, at later time points at least, there are defects in osteoblastogenic potential of marrow stromal cells after SCI, which resemble those observed with tail suspension.

The finding that osteoblastogenic differentiation was not altered by 7 days of ES was not expected and contrasts with the normalization of osteoblastogenic potential of bone marrow stromal cells after resuming bone loading following 2 weeks of tail suspension (41, 42). A few possible mechanisms may be considered to explain this surprising finding. For example, prolonged periods of immobilization may result in fundamental changes in the pool of osteoblast precursors available that are irreversible or require longer or larger stimuli to overcome; the physiologic changes after SCI may have unique and more severe

FIGURE 5. The changes of gene expression and signaling pathway in ex vivo cultured osteoclasts. A, mRNA levels were determined by real time PCR. n = 3–4 per group. * , p < 0.05; ** , p < 0.01, and *** , p < 0.001 versus indicated group. B, a pathways analysis of differentially expressed genes identified by Illumina microarray analysis was performed. Selected gene expression changes were also highlighted.
effects on the marrow stromal cell populations or progenitors committed to the osteoblast lineage that require additional anabolic challenges before their adverse influence is overcome. Even so, ES stimulated several changes in gene expression in *ex vivo* cultures of osteoblasts that indicate changes in the biology of the cells from which they were derived conducive to a shift away from sustained net bone resorption toward greater net bone formation. These include increased OPG mRNA levels and reduced expression of the Wnt inhibitors DKK1 and sFRP2.

**Effects of SCI- and ES-induced Muscle Contraction on Wnt Signaling**—Analysis of biological themes represented by the gene expression data for osteoblast cultures from SCI-ES animals identified Wnt signaling as a critical pathway regulated by ES in these cells. Wnt signaling is essential in commitment of mesenchymal precursor cells to the osteoblast lineage, to osteoblastic differentiation, and to stimulating osteoblasts to form new bone (11). Release of RANKL by cells of the osteoblast/osteocyte lineage also appears to be regulated by Wnt signaling in a load-dependent manner; sclerostin stimulates release of RANKL by osteocytes, thus initiating a signaling cascade driving bone resorption (14). Osteocyte expression of sclerostin is up-regulated by unloading, and a neutralizing antibody against sclerostin has been found to inhibit bone loss due to hindlimb unloading and to normalize osteoblastic differentiation of marrow stromal cells *in vitro* (43). Although our findings do not permit a firm conclusion, several gene expression changes suggest that ES stimulated a net increase in Wnt signaling in bone marrow cell-derived osteoblasts. The most persuasive findings are an increase in the expression of three genes that have been reported to be up-regulated by Wnt signaling: Runx2 (44), OPG (18), and *Enc1* (29). In addition, ES increased Wnt3a expression and normalized the expression of the inhibitors of Wnt signaling DKK1 and sFRP2, the expression of which was increased by SCI. Interestingly, *Enc1* has recently been suggested to be a novel candidate protein for osteoblast differentiation.7 Of note, we recently reported that when administered to rats with SCI, nandrolone, a synthetic androgen, increased bone mass and expression in osteoblasts derived from bone marrow stromal cells of the osteoblast differentiation markers Runx2 and osteocalcin and reversed the SCI-induced inhibition of *Enc1* expression (Sun et al. (62)). These data also support a relationship between greater Wnt signaling, increased *Enc1* expression, and improved osteoblast differentiation. Taking these data together, it is both attractive and logical to propose that reloading of bone by ES elicits significant increases in Wnt signaling in bone of SCI animals. One logical direction for future studies evaluating the contribution of Wnt signaling to ES-induced changes in bone could include a targeted deletion of Wnt-related genes (*e.g.* β-catenin) in osteoblasts or osteocytes.

Given the weight of evidence supporting an overall increase in Wnt signaling in cultures of osteoblasts derived from marrow of SCI-ES animals, a surprising finding was that ES only partially reduced SOST expression, whereas ES diminished expression of Dkk1 and sFRP2 to levels observed in sham-SCI rats. The reasons for this divergence in expression of Wnt inhibitors are not clear. It is also interesting that SOST is expressed in these cultures because SOST is primarily expressed in osteocytes. One possible explanation is that bone nodules do contain a subpopulation of cells with properties similar to osteocytes; consistent with this view, at 15 days of culture, when alkaline phosphatase-positive cells are present in cultures but bone nodules have not yet been formed, SOST expression is undetectable.8

The increased expression of SOST observed in cultured osteoblasts at 17 weeks after SCI was similar to findings of increased expression of SOST observed in osteocytes in other models of bone unloading (45) and is in general agreement with increased blood levels of sclerostin in immobilized patients (46). These findings suggest that changes in SOST expression are similar across states of bone unloading. The up-regulation of Dkk1 observed in bone marrow-derived osteoblasts from SCI rats was not an anticipated finding, but not totally unexpected. Dkk1 has been found to be expressed at increased levels after unloading for 1 day in mice (12), and it has been implicated in bone loss due to glucocorticoids and menopause (47). The up-regulation in bone of the expression of mRNA for SOST, Dkk1, and sFRP2 after SCI raises the question of whether a common, load-dependent mechanism controls their expression in osteoblasts. If this proves to be the case, additional mechanisms for fine-tuning such a common mechanism for co-regulation of Wnt inhibitors must exist to explain the differential response of these genes to a short period of reloading. One might also ask whether there is a sequence in which the expression of Wnt inhibitors or Wnts is altered after unloading or reloading, and if so, how this temporal regulation occurs. There is a possibility that these Wnt inhibitors themselves participate in such regulation. Evidence of such interactions has been presented in studies of a model of bone loss due to inflammation in which it was reported that antibodies against DKK1 prevented TNF-α-induced up-regulation of sclerostin expression (48).

One of the interesting findings in this study is that Wnt5a, which has been reported to oppose the actions of Wnts acting through canonical Wnt/β-catenin signaling such as Wnt3a (49), was significantly up-regulated in the cultured osteoblasts from SCI rats. A recent study demonstrated that Wnt5a, via noncanonical Wnt signaling, enhanced RANKL-induced osteoclast formation in mouse bone marrow macrophage cultures (50, 51). Thus, it is possible that overproduction of Wnt5a in osteoblasts after SCI plays dual roles in unloading-related bone loss by compromising the beneficial role of increased Wnt3a on bone formation after SCI and promoting osteoclastogenesis and bone resorption by increasing RANKL production.

**Effects of SCI- and ES-induced Muscle Contraction on Other Signaling Pathways**—The pathways analysis of mRNA expression changes in osteoblasts at 3 months after SCI found alterations in gene expression consistent with reduced signaling by BMPs, TGF-β and IGF-1 (52). At 17 weeks after SCI, we found that in *ex vivo* cultures of osteoclasts, enriched pathways included PTH, vitamin D, oxytocin, FSH, and calcineurin signaling, whereas in osteoblasts, Hedgehog-PTH, Wnt, calcineu-

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7 J. Whitehead, personal communication.

8 W. Qin, unpublished observations.
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rin signaling, and β-adrenergic signaling were among the enriched pathways observed. How each of these alterations promotes bone loss after unloading, or bone responses to reloading, is not known, but a growing literature supports a role for each of these pathways in bone biology (16). The roles of β-adrenergic signaling will be discussed in the next section. PTH is a pro-resorptive hormone, but is also anabolic to bone when administered in a pulsatile manner (16). The calcium-dependent activation of calcineurin-calmodulin complexes plays important roles in bone formation and resorption by osteoblasts and osteoclasts, respectively (53). The transcription factor Mef2c is a downstream effector of calcineurin/NFAT signaling that controls bone development by activating the gene program for chondrocyte hypertrophy (54). The role of other isoforms of the Mef2 family in bone metabolism has not been established. ES reversed SCI-mediated inhibition of the expression of Mef2d in osteoblasts. It may be that this effect of ES is important to effects of ES on osteoblast biology; the precise significance of this change is as yet uncertain.

ES reduced expression of Smad3, a transcriptional regulator that is a downstream effector of receptors for activins, myostatin, and GDF11 (55). Smad3 is important for RANKL-induced osteoclastogenic signaling, and activins have been reported to reduce bone mass in adulthood (56). Oxytocin has been found to be anabolic to bone by stimulating differentiation of osteoblasts to a mineralizing phenotype and by inhibiting bone resorption by mature osteoclasts (57). Future investigation would be of interest to study how each of these alterations coordinates and contributes to the beneficial effects of ES-induced muscle contraction on bone after SCI.

Effects of Reloading by ES-induced Muscle Contraction Are Independent of the Higher CNS—The effects of mechanical reloading of bone by ES on bone metabolism and bone cells occurred despite complete interruption of all afferent and efferent connections between bone and higher centers of the CNS. Innervation of the femur and tibia includes parasympathetic efferents that arise from the sacral spinal cord as well as motor, sensory, and sympathetic fibers (58). Thus, the SCI model used in our study permits one to test whether neural signals orchestrated by the brain are necessary for osseous responses to reloading of bone and could be used to study the role of neural inputs arising from the brain on bone biology.

The sympathetic nervous system has been found to modulate bone through β2-adrenergic receptors (Aдрb2) with leptin and neuromedin U, playing key roles in the central control of sympathetic nervous system activity (4, 5, 59, 60). In mice, increased tone of the sympathetic nervous system has been suggested to be responsible for reduced bone formation in a mouse model of immobilization through activation of Aдрb2 expressed in bone marrow cell-derived osteoblasts (61). However, our current findings indicate that neural signals from the brain are not critical to bone responses to resumption of neuromuscular activity and suggest that mechanical loading, possibly together with altered expression of myokines, is sufficient to reduce bone resorption and stimulate changes in osteoblasts independently of the brain. Interestingly, we found that ES reversed SCI-mediated inhibition of Aдрb2 and up-regulation of Aдрb3 in bone marrow stem cell-derived osteoblasts. The findings might suggest local effects of ES on adrenergic receptors present in osteogenic cells (59), which needs to be further investigated.

Our studies do not permit separation of possible local neural effects of ES, such as retrograde conduction of stimuli in sensory nerves, from those of the purely mechanical effects on bone of muscle contraction or humoral effects due to resumption of neuromuscular activity. Skeletal muscle is also richly innervated by the sensory and autonomic nervous systems, and the SCI model used in our studies may also provide insights as to how higher centers of the central nervous system influence skeletal muscle. Also important is that through neural inputs to skeletal muscle and bone, higher centers of the CNS may fine-tune humoral interactions between them.

Conclusions—In the present study, we found that after a 17-week period of extreme unloading due to spinal cord transection, bone resorption was elevated and was greatly reduced by 7 days of reloading by ES-induced muscle contraction. In ex vivo cultures of bone marrow hematopoietic cells, ES normalized the otherwise elevated number of osteoclasts observed; in cultures of bone marrow stromal cells differentiated into osteoclasts, favorable gene expression changes were observed that were indicative of increased Wnt signaling and ratio of OPG/RANKL mRNA levels. Importantly, these rapid cellular and molecular responses occurred independently of higher CNS influence. Microarray analysis also suggests that the involvement of signaling through PTH, FSH, oxytocin, and calcineurin/NFAT may be involved in bone resorption resulting from unloading and/or effects of reloading to prevent it. To our knowledge, this is the first description of the metabolic, cellular, or gene expression changes in response to bone reloading after extreme unloading (e.g., SCI) when begun after an extended period of unloading and the first detailed assessment of global changes in mRNA transcript levels after bone reloading induced by muscle contraction in any setting of unloading-related bone loss. The study provides insight into cellular and molecular mechanisms regarding the biological effects of muscle reloading of bone.

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REFERENCES

1. Schoenau, E. (2005) From mechanostat theory to development of the “Functional Muscle-Bone-Unit”. J. Musculoskelet. Neuronal Interact. 5, 232–238
2. Rittweger, J., Beller, G., Ehrig, J., Jung, C., Koch, U., Ramolla, J., Schmidt, F., Newitt, D., Majumdar, S., Schiessl, H., and Felsenberg, D. (2000) Bone-muscle strength indices for the human lower leg. Bone 27, 319–326
3. Dudley-Javoroski, S., and Shields, R. K. (2008) Muscle and bone plasticity after spinal cord injury: review of adaptations to disuse and to electrical muscle stimulation. J. Rehabil. Res. Dev. 45, 283–296
4. Qin, W., Bauman, W. A., and Cardozo, C. (2010) Bone and muscle loss after spinal cord injury: organ interactions. Annu. N.Y. Acad. Sci. 1211, 66–84
5. Qin, W., Bauman, W. A., and Cardozo, C. P. (2010) Evolving concepts in neurogenic osteoporosis. Curr. Osteoporos. Rep. 8, 212–218
6. Thomaidis, V. T., and Lindholm, T. S. (1976) The effect of remobilisation on the extremity of the adult rat after short-term immobilisation in a plaster cast. Acta Chir. Scand. Suppl. 467, 36–39
7. Lepola, V., Väänänen, K., and Jalovaara, P. (1993) The effect of immobili-
zation on the torsional strength of the rat tibia. *Clin. Orthop. Relat. Res.* 55–61.

8. Tuukkanen, J., Wallmark, B., Jalovaara, P., Takala, T., Sjögren, S., and Väänänen, K. (1991) Changes induced in growing rat bone by immobilization and remobilization. *Bone* 12, 113–118.

9. Lang, T. F., Leblanc, A. D., Evans, H. J., and Lu, Y. (2006) Adaptation of the proximal femur to skeletal reloading after long-duration spaceflight. *J. Bone Miner. Res.* 21, 1224–1230.

10. Bonevaid, L. F., and Johnson, M. L. (2008) Osteocytes, mechanosensing, and Wnt signaling. *Bone* 42, 606–615.

11. Kubota, T., Michigami, T., and Ozono, K. (2009) Wnt signaling in bone metabolism. *J. Bone Miner. Metab.* 27, 265–271.

12. Robling, A. G., Nizolek, P. J., Baldridge, L. A., Condon, K. W., Allen, M. R., Alam, I., Mantilla, S. M., Ghukh-Hinrich, J., Bellido, T. M., Harris, S. E., and Turner, C. H. (2008) Mechanical stimulation of bone *in vivo* reduces osteocyte expression of Sost/sclerostin. *J. Biol. Chem.* 283, 5866–5875.

13. Xiong, J., Oral, M., Jäla, R. L., Weinstein, R. S., Manolagas, S. C., and O’Brien, C. A. (2011) Matrix-embedded cells control osteostal formation. *Nat. Med.* 17, 1235–1241.

14. Wijenayaka, A. R., Kogawa, M., Lim, H. P., Bonewald, L. F., Findlay, D. M., Kearns, A. E., Khosla, S., and Kostenuik, P. J. (2008) Receptor activator of nuclear factor-κB ligand and osteoprotegerin regulation of bone remodeling in health and disease. *Endocr. Rev.* 29, 155–192.

15. Zaidi, M. (2007) Skeletal remodeling in health and disease. *Nat. Med.* 13, 791–801.

16. Tu, X., Rhee, Y., Condon, K. W., Bivi, N., Allen, M. R., Dwyer, D., Stolina, M., Turner, C. H., Robling, A. G., Plotkin, L. I., and Bellido, T. (2012) Sost downregulation and local Wnt signaling are required for the osteogenic response to mechanical loading. *Bone* 50, 209–217.

17. Glass, D. A., 2nd, Bialek, P., Ahn, J. D., Starbuck, M., Patel, M. S., Clevers, H., Taketo, M. M., Long, F., McMahon, A. P., Lang, R. A., and Karsenty, G. (2005) Canonical Wnt signaling in differentiated osteoblasts controls osteoclast differentiation. *Dev. Cell* 8, 751–764.

18. Wu, Y., Zhao, J., Zhao, W., Pan, J., Bauman, W. A., and Cardozo, C. P. (2012) Nandrolone normalizes determinants of muscle mass and fiber type after spinal cord injury. *J. Neurotrauma* 29, 1663–1675.

19. Parfitt, A. M., Khosla, S., and Kostenuik, P. J. (2008) Receptor activator of nuclear factor κB ligand and osteoprotegerin regulation of bone remodeling in health and disease. *Endocr. Rev.* 29, 155–192.

20. Qin, W., Pan, J., Wu, Y., Bauman, W. A., and Cardozo, C. (2010) Protection against demethasone-induced muscle atrophy. *Calcif. Tissue Int.* 87, 596.

21. A. J., Sawitzki, G., Smith, C., Smyth, G., Tierney, L., Yang, J. Y., and Zhang, J. (2004) Bioconductor: open software development for computational biology and bioinformatics. *Genome Biol.* 5, R80.

22. Livak, K. J., and Schmittgen, T. D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2 ΔΔCT method. *Methods* 25, 402–408.

23. Fujita, M., Furukawa, Y., Tsumoda, T., Tanaka, T., Ogawa, M., and Nakamura, Y. (2001) Up-regulation of the ectodermal-neural crest 1 (EN1) gene, a downstream target of the β-catenin/T cell factor complex, in colorectal carcinomas. *Cancer Res.* 61, 7722–7726.

24. Leupin, O., Kramer, I., Collette, N. M., Loos, G. G., Natt, F., Kneissel, M., and Keller, H. (2007) Control of the SOST bone enhancer by PTH using MEF2 transcription factors. *J. Bone Miner. Res.* 22, 1957–1967.

25. Yasui, T., Kadono, Y., Nakamura, M., Oshima, Y., Matsumoto, T., Masuda, H., Hirose, J., Omata, Y., Yasuda, H., Immamura, T., Nakamura, K., and Tanaka, S. (2011) Regulation of RANKL-induced osteoclastogenesis by TGF-β through molecular interaction between Smad3 and Traf6. *Bone Miner. Res.* 26, 144–156.

26. Pearman, A. T., Chou, W. Y., Bergman, K. D., Pulumati, M. R., and Partridge, N. C. (1996) Parathyroid hormone induces c-fos promoter activity in osteoblastic cells through phosphorylated cAMP response element (CRE)-binding protein binding to the major CRE. *J. Biol. Chem.* 271, 25715–25721.

27. Grigoriadis, A. E., Wang, Z. Q., Cecchinii, M. G., Hofstetter, W., Felix, R., Fleisch, H. A., and Wagner, E. F. (1994) c-Fos: a major regulator of osteoclastogene-macrophage lineage determination and bone remodeling. *Science* 266, 443–448.

28. Abreu, E. L., Stern, M., and Brocco, M. (2012) Bone–muscle interactions: ASBMR Topical Meeting, July 2012. *IBMS BoneKEy* 10.1038/bonekey.2012.239.

29. Mo, C., Romero-Suarez, S., Bonevaid, L., Johnson, M., and Brocco, M. (2012) Prostaglandin E2: from clinical applications to its potential role in bone-muscle crosstalk and myogenic differentiation. *Recent Pat. Biotechnol.* 6, 223–229.

30. Hamrick, M. W., and Ferrari, S. L. (2008) Leptin and the sympathetic connection of fat to bone. *Osteoporos Int.* 19, 905–912.

31. Jahn, K., Lara-Castillo, N., Brocco, L., Mo, C. L., Johnson, M. L., Brocco, M., and Bonevaid, L. F. (2012) Skeletal muscle secreted factors prevent glucocorticoid-induced osteocyte apoptosis through activation of β-catenin. *Eur. Cell. Mater.* 24, 197–209.

32. Hafer-Macko, C. E., Yu, S., Ryan, A. S., Ivey, F. M., and Macko, R. F. (2005) Elevated tumor necrosis factor-α in skeletal muscle after stroke. *Stroke* 36, 2021–2023.

33. Ryan, A. S., Ivey, F. M., Prior, S., Li, G., and Hafer-Macko, C. (2011) Skeletal muscle hypertrophy and muscle myostatin reduction after resistive training in stroke survivors. *Stroke* 42, 416–420.

34. Sessions, N. D., Halloran, B. P., Bikle, D. D., Wronski, T. J., Cone, C. M., and Morey-Holton, E. (1989) Bone response to normal weight bearing after a period of skeletal unloading. *Am. J. Physiol.* 257, E606–E610.

35. Basso, N., Jia, Y., Bellows, C. G., and Heersche, J. N. (2005) The effect of reloading on bone volume, osteoblast number, and osteoprogenitor characteristics: studies in hind limb unloaded rats. *Bone* 37, 370–378.

36. Zhang, R., Supowit, S. C., Klein, G. L., Lu, Z., Christensen, M. D., Lozano, R., and Simmons, D. J. (1995) Rat tail suspension reduces messenger RNA level for growth factors and osteopontin and decreases the osteoblastic differentiation of bone marrow stromal cells. *J. Bone Miner Res.* 10, 415–423.

37. Shahnazari, M., Wronski, T., Chu, V., Williams, A., Leeper, A., Stolina, M., Ke, H. Z., and Hallorain, B. (2012) Early response of bone marrow osteo-progenitors to skeletal unloading and sclerostin antibody. *Calcif. Tissue Int.* 91, 50–58.

38. Gaur, T., Lengner, C. J., Hovhannisian, H., Bhat, R. A., Bodine, P. V., Komr, B. S., Javed, A., van Wijnen, A. J., Stein, J. L., Stein, G. S., and Lian, J. B. (2005) Canonical WNT signaling promotes osteogenesis by directly stimulating Runx2 gene expression. *J. Biol. Chem.* 280, 33132–33140.

39. Lin, C., Jiang, X., Dai, Z., Guo, X., Weng, T., Wang, J., Li, Y., Feng, G., Gao, X., and He, L. (2009) Sclerostin mediates bone response to mechanical unloading through antagonizing Wnt/β-catenin signaling. *J. Bone Miner. Res.*
46. Gaudio, A., Pennisi, P., Bratengeier, C., Torrisi, V., Lindner, B., Mangiafico, R. A., Pulvirenti, I., Hawa, G., Tringali, G., and Fiore, C. E. (2010) Increased sclerostin serum levels associated with bone formation and resorption markers in patients with immobilization-induced bone loss. *J. Clin. Endocrinol. Metab.* 95, 2248–2253

47. Pinzone, J. J., Hall, B. M., Thudi, N. K., Vonau, M., Qiang, Y. W., Rosol, T. J., and Shaughnessy, J. D., Jr. (2009) The role of Dickkopf-1 in bone development, homeostasis, and disease. *Blood* 113, 517–525

48. Heiland, G. R., Zwerina, K., Baum, W., Kireva, T., Distler, J. H., Grisanti, M., Asuncion, F., Li, X., Ominsky, M., Richards, W., Schett, G., and Zwerina, J. (2010) Neutralisation of Dkk-1 protects from systemic bone loss during inflammation and reduces sclerostin expression. *Ann. Rheum. Dis.* 69, 2152–2159

49. Yuan, Y., Niu, C. C., Deng, G., Li, Z. Q., Pan, J., Zhao, C., Yang, Z. L., and Si, W. K. (2011) The Wnt5a/Ror2 noncanonical signaling pathway inhibits canonical Wnt signaling in K562 cells. *Int. J. Mol. Med.* 27, 63–69

50. Maeda, K., Kobayashi, Y., Udagawa, N., Uehara, S., Ishihara, A., Mizoguchi, T., Kikuchi, Y., Takada, I., Kato, S., Kani, S., Nishita, M., Marumo, K., Martin, T. J., Minami, Y., and Takahashi, N. (2012) Wnt5a-Ror2 signaling between osteoblast-lineage cells and osteoclast precursors enhances osteoclastogenesis. *Nat. Med.* 18, 405–412

51. Takahashi, N., Maeda, K., Ishihara, A., Uehara, S., and Kobayashi, Y. (2011) Regulatory mechanism of osteoclastogenesis by RANKL and Wnt signals. *Front. Biosci.* 16, 21–30

52. Jiang, S. D., Yan, J., Jiang, L. S., and Dai, L. Y. (2011) Down-regulation of the Wnt, estrogen receptor, insulin-like growth factor-I, and bone morphogenetic protein pathways in osteoblasts from rats with chronic spinal cord injury. *Joint Bone Spine* 78, 488–492

53. Sitara, D., and Aliprantis, A. O. (2010) Transcriptional regulation of bone and joint remodeling by NFAT. *Immunol. Rev.* 233, 286–300

54. Arnold, M. A., Kim, Y., Czubryt, M. P., Phan, D., McAnally, J., Qi, X., Shelton, J. M., Richardson, J. A., Bassel-Duby, R., and Olson, E. N. (2007) MEF2C transcription factor controls chondrocyte hypertrophy and bone development. *Dev. Cell* 12, 377–389

55. Andersson, O., Reissmann, E., and Ibáñez, C. F. (2006) Growth differentiation factor 11 signals through the transforming growth factor-β receptor ALK5 to regionalize the anterior-posterior axis. *EMBO Rep.* 7, 831–837

56. Chantry, A. D., Heath, D., Mulivor, A. W., Pearsall, S., Baud'huin, M., Coulton, L., Evans, H., Abdul, N., Werner, E. D., Bouxsein, M. L., Key, M. L., Sehra, J., Arnett, T. R., Vanderkerken, K., and Croucher, P. (2010) Inhibiting activin-A signaling stimulates bone formation and prevents cancer-induced bone destruction in vivo. *J. Bone Miner. Res.* 25, 2633–2646

57. Tamma, R., Colaianni, G., Zhu, L. L., DiBenedetto, A., Greco, G., Montemurro, G., Patano, N., Strippoli, M., Vergari, R., Mancini, L., Colucci, S., Grano, M., Faccio, R., Liu, X., Li, J., Usmani, S., Bachar, M., Bab, I., Nishimori, K., Young, L. J., Buettner, C., Iqbal, J., Sun, L., Zaidi, M., and Zallone, A. (2009) Oxytocin is an anabolic bone hormone. *Proc. Natl. Acad. Sci. U.S.A.* 106, 7149–7154

58. Krassioukov, A. V., Karlsson, A. K., Wecht, J. M., Wuermser, L. A., Mathias, C. J., and Marino, R. J. (2007) Assessment of autonomic dysfunction following spinal cord injury: rationale for additions to International Standards for Neurological Assessment. *J. Rehabil. Res. Dev.* 44, 103–112

59. Takeda, S., Elefteriou, F., Levasseur, R., Liu, X., Zhao, L., Parker, K. L., Armstrong, D., Ducy, P., and Karsenty, G. (2002) Leptin regulates bone formation via the sympathetic nervous system. *Cell* 111, 305–317

60. Sato, S., Hanada, R., Kimura, A., Abe, T., Matsumoto, T., Iwasaki, M., Inose, H., Ida, T., Mieda, M., Takeuchi, Y., Fukushima, S., Fujita, T., Kato, S., Kangawa, K., Kojima, M., Shinomiya, K., and Takeda, S. (2007) Central control of bone remodeling by neuropeptides. *Nat. Med.* 13, 1234–1240

61. Kondo, H., Nifuji, A., Takeda, S., Ezura, Y., Ritting, S. R., Denhardt, D. T., Nakashima, K., Karsenty, G., and Noda, M. (2005) Unloading induces osteoblastic cell suppression and osteoclastic cell activation to lead to bone loss via sympathetic nervous system. *J. Biol. Chem.* 280, 30192–30200

62. Sun, L., Pan, J., Peng, Y., Wu, Y., Li, J., Liu, X., Qin, Y., Bauman, W. A., Cardozo, C., Zaidi, M., and Qin, W. (2012) Anabolic steroids reduce bone loss due from spinal cord injury associated with increased Wnt signaling. *J. Spinal Cord Med.*, in press