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# Periostin expression contributes to cortical bone loss during unloading

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ABSTRACT

Periostin (a product of *Postn* gene) is a matricellular protein which is increased in periosteal osteoblasts and osteocytes upon mechanical stimulation. We previously reported that periostin-deficient mice ($Postn^{-/-}$) have low bone mass and a diminished response to physical activity due to a lack of sclerostin (a product of *Sost* gene) inhibition by mechanical loading. Here we hypothesized that periostin could play a central role in the control of bone loss during unloading induced by hindlimb suspension (HU).

In $Postn^{+/+}$ mice (wildtype littermate), HU significantly decreased femur BMD, as well as trabecular BV/TV and thickness (Tb.Th). Cortical bone volume and thickness at the femoral midshaft, also significantly decreased. These changes were explained by an inhibition of endocortical and periosteal bone formation activity and correlated with a decrease of *Postn* expression and a consecutive increase in *Sost* early after HU. Whereas trabecular bone loss in $Postn^{-/-}$ mice was comparable to $Postn^{+/+}$ mice, HU did not significantly alter cortical bone microstructure and strength in $Postn^{-/-}$ mice. Bone formation remained unchanged in these mice, as *Sost* did not increase in the absence of periostin. In contrast, changes in *Dkk1*, *Rankl* and *Opg* expression in response to HU were similar to $Postn^{+/+}$ mice, indicating that changes in periostin expression were quite specifically related to changes in *Sost*. In conclusion, HU inhibits periostin expression, which in turn plays an important role in cortical bone loss through an increase in *Sost*. These results further indicate that periostin is an essential mediator of cortical bone response to mechanical forces (loading and unloading).



## Introduction

Weightlessness and immobility, as experienced by bedridden, immobilized patients or astronauts, lead to a reduction in bone mass and strength. Skeletal adaptation to unloading involves a continuous reduction in bone formation rate but also a precocious and transient increase in bone resorption [1,2]. Osteocytes orchestrate mechanotransduction in bone, as their ablation diminishes the bone response to loading [3]. Among the osteocytic molecules involved in mechanotransduction, sclerostin plays a major role through the inhibition of Wnt/β-catenin signaling [4–7]. Hence sclerostin gene (*Sost*) expression is inhibited by loading, which leads mainly but not exclusively to an increase in bone formation [8,9]. In accordance, SOST expression increases with unloading [8]. In addition to its prominent inhibitory effects on bone formation, some *in vitro* and *in vivo* studies suggest that sclerostin also stimulates bone resorption by decreasing the OPG/RANKL ratio in osteoblasts and osteocytes [9]. Thus, Sost-deficient mice are resistant to bone loss induced by hindlimb suspension, although the contribution of bone formation and/or bone resorption in this model remains unclear [5]. Accordingly, pharmacological inhibition of sclerostin restores bone formation in different models of microgravity, i.e., tail suspension, immobilized rats, and spine injury model [10–13]. We previously reported that down regulation of SOST and cortical bone formation in response to mechanical loading and parathyroid hormone (PTH) depends on the expression of a matricellular protein, periostin, the expression of which is increased in these conditions [14,15]. In turn, periostin triggers β-catenin mediated signaling [15]. Noteworthy, β-catenin mediated the stimulation of OPG expression in osteocytes [16], raising the possibility that periostin could also be involved in the control of bone resorption by these cells. In addition, periostin is implicated in tissue regeneration and repair mechanisms, which has been previously described in the lungs and heart [17]. Besides, periostin expression is increased by inflammation and mechanical stress, suggesting a potential function of this molecule in maintaining the structure and integrity of connective tissues. Periostin binds to integrins αvβ3 and αvβ5, regulating cell adhesion and mobility [18,19] and promotes cell survival via the Akt/protein kinase B pathway. These observations clearly suggest that periostin could be a key role in skeletal adaptation to unloading. We hypothesized that unloading would reduce periostin expression, which in turn might contribute to increasing Sost and decreasing OPG, and thereby control the detrimental effects of unloading on bone. More specifically, in the absence of periostin,

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bone mineral density (BMD), structure and bone formation decrease would be attenuated.

In order to test this hypothesis, we characterized bone mass, microarchitecture and strength in response to 7 and 21 days of hindlimb unloading (HU) in *Postn*$^{-/-}$ and *Postn*$^{+/+}$ mice. In *Postn*$^{+/+}$, cortical and trabecular bone loss were proportionated, whereas in *Postn*$^{-/-}$ bone loss occurred exclusively in the trabecular compartment. In *Postn*$^{+/+}$, *Postn* mRNA decreased at 7 and 21 days, whereas Sost increased only at 7 days with unloading. These changes were paralleled by a sustained inhibition of bone formation, particularly in the cortical compartment, and also by a transient increase in osteoclast number. In the absence of periostin regulation by unloading in *Postn*$^{-/-}$ mice, cortical bone formation was maintained, especially in the periosteum.

## Materials and methods

### *Animals*

To generate *peri*$^{lacZ}$ knock-in mice, Rios et al. generated a replacement-targeting vector, where the bacterial β-galactosidase gene was knocked into the *periostin* gene locus [20], so that the Lac Z reporter was expressed instead of periostin under the control of the periostin promoter. They infected 129SvJ mouse embryonic stem (ES) with this vector and generated chimeras by microinjection of ES using standard protocols. Chimeras were subsequently bred with C57BL/6 to generate heterozygotes. These were subsequently backcrossed for 10 generations, resulting in a genome of 99% C57BL/6J. Mice were singly housed 2 weeks before initiation of unloading, maintained under standard conditions and had access to water and soft diet *ad libitum* (Harlan Teklad 2019, SDS, England). Soft diet had been chosen to reduce the malnutrition of the *Postn*$^{-/-}$ mice observed under standard diet due to the enamel and dentin defects of the incisors and molars [20].

Three-month-old male *Postn*$^{-/-}$ and *Postn*$^{+/+}$ mice were subjected to 7 or 21 days of unloading ($n = 8$ per genotype for unloading group and $n = 6$ for controls). HU was achieved by tail suspension as previously described [2]. The height of the mice hindquarters was adjusted to prevent any contact of the hindlimbs with the cage floor, resulting in approximately a 30° head-down tilt. The forelimbs of the animals maintained contact with the cage bottom, allowing the mice full access to the entire cage. Normal weight-bearing mice (Control) were also singly housed.

To measure dynamic indices of bone formation, mice received subcutaneous injections of calcein (25 mg/kg, Sigma, Buchs, Switzerland) 7 and 2 days before euthanasia. Animal procedures were approved by the University of Geneva School of Medicine Ethical Committee and the State of Geneva Veterinary Office.

### *In vivo measurement of BMD*

Total body, femoral and spinal BMD (g/cm$^2$) were measured *in vivo* by dual-energy X-ray absorptiometry (PIXImus2, GE lunar, Madison WI) [21] before initiation of tail suspension and just before euthanasia, i.e., after 7 and 21 days of unloading.

### *Ex vivo measurement of microarchitecture*

Micro-computed tomography (microCT UCT40, Scanco Medical AG, Basserdorf Switzerland) was used to assess trabecular bone volume fraction in the distal femur, and cortical bone geometry at the midshaft femur diaphysis, as previously described [14]. Briefly, trabecular and cortical bone regions were evaluated using isotropic 12 μm voxels.

For the trabecular region, to eliminate the primary spongiosa, we analyzed 100 slices starting from 50 slices below the distal growth plate.

Femoral cortical geometry was assessed using 50 continuous CT slides (600 μm) located at the midshaft. Images were segmented using a fixed threshold approach. Morphometric variables were computed from binarized images using direct, three-dimensional techniques that do not rely on prior assumptions about the underlying structure [22]. For the trabecular bone regions, we assessed bone volume fraction (BV/TV, %), trabecular thickness (TbTh, μm), trabecular number (TbN, mm$^{-1}$), trabecular connectivity density (Tb Conn Density, mm$^{-3}$), and structural model index (SMI). The latter was measured to determine the prevalence of plate-like or rod-like trabecular structures, where 0 represents "plates" and 3 "rods" [22]. For cortical bone at the femoral midshaft, we measured cortical tissue volume (CtTV, mm$^3$), bone volume (CtBV, mm$^3$), marrow volume (BMaV, mm$^3$), and average cortical width (CtTh, μm).

### *RNA extraction and quantitative PCR*

The whole tibia was excised; both tibial extremities were cut and diaphysis was flushed with cold PBS to separate the bone marrow from the cortex. Tibial diaphyses were immediately pulverized to a fine powder and homogenized in peqGold Trifast (peQLab Biotechnologie GmbH) using FastPrep System apparatus (QBiogene) in order to achieve quantitative RNA extraction. Total RNA was extracted and then purified on mini-columns (RNeasy Mini kit, Qiagen) in combination with a deoxyribonuclease treatment (RNase-free DNase Set, Qiagen) to avoid DNA contamination.

Single stranded cDNA templates for quantitative real-time PCR (qRT-PCR) analyses were carried out using SuperScript III Reverse Transcriptase (Invitrogen AG, Basel) following the manufacturer's instructions. Quantitative RT-PCR was performed using predesigned TaqMan® Gene Expression Assays (references in Table S1). A Biomek 2000 robot (Beckman Coulter, Nyon, Switzerland) was used for liquid handling (10 μl) in 384-well plates with 3 replicates per sample. The cDNA was PCR amplified in a 7900HT SDS System and raw threshold-cycle (Ct) values were obtained from SDS 2.0 software (Applied Biosystems, Rotkreuz, Switzerland). Relative quantities (RQ) were calculated with the formula $RQ = E - Ct$ using an efficiency (E) of 2 by default. For each gene the highest quantity was arbitrarily designated a value of 1.0. The mean quantity was calculated from triplicates for each sample and this quantity was normalized to the similarly measured mean quantity of the *GAPDH* normalization gene. Finally, normalized quantities were averaged to 4 animals and represented as mean ± SEM (Table S1).

### *Histomorphometry*

To measure dynamic indices of bone formation, mice received subcutaneous injections of calcein (10 mg/kg, Sigma, Buchs, Switzerland) 7 and 2 days before euthanasia. Femurs were embedded in methyl methacrylate (Merck, Switzerland), and 8-μm-thick transversal sections of the midshaft were cut with a Polycot E microtome (Leica Corp. Microsystems AG, Glattburg, Switzerland) and mounted unstained for evaluation of fluorescence. 5-μm thick sagittal sections were stained with modified Goldner's trichrome, and histomorphometric measurements were performed on the secondary spongiosa of the proximal tibia metaphysis and on the endocortical and periosteal bone surfaces in the middle of the tibia, using a Leica Corp. Q image analyzer at 40× magnification. All parameters were calculated and expressed according to standard formulas and nomenclatures [23]: mineral apposition rate (MAR, μm/day), single labeled surface (sLS/BS, %), and double-labeled surface (dLS/BS, %), mineralizing perimeter per bone perimeter (MPm/BPm, %). Mineralizing surface per bone surface (MS/BS, %) was calculated by adding dLS/BS and one-half sLS/BS. Bone formation rate (BFR/BS, μm$^3$/μm$^2$/day) was calculated as the product of MS/BS and MAR.

TRAP was detected by using hexazotized pararosanilin (Sigma, St Louis, MO) and naphtol ASTR phosphate (Sigma, St Louis, MO) to reveal osteoclasts; non-osteoclastic acid phosphatase was inhibited by adding 100 mMol/l L(+)-tartric acid (Sigma, St Louis, MO) to