1 **Title:** Changes in the intra- and peri-cellular sclerostin distribution in lacuno-canalicular system

2 induced by mechanical unloading

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Abstract

Background: Mechanical stimuli regulate Sclerostin (Scl), a negative regulator of bone formation, expression in osteocytes. However, the detailed Scl distribution in osteocytes in response to mechanical unloading remains unclear.

Methods: Twelve-week-old male rats were used. The sciatic and femoral nerves on the right side were excised as mechanical unloading treatment. A sham operation was performed on the left side. One week after neurotrauma, the bone density of the femora was evaluated by peripheral quantitative computed tomography, and immunofluorescence was performed in coronal sections of the femoral diaphysis. The mean fluorescence intensity and fluorescent profile of Scl from the marrow to the periosteal side were analyzed to estimate the Scl expression and determine to which side (marrow or periosteal) the Scl prefers to distribute in response to mechanical unloading. The most sensitive region indicated by the immunofluorescence results was further investigated by transmission electron microscopy (TEM) with immunogold staining to show the Scl expression changes in different subcellular structures.

Results: In femur distal metaphysis, neurotrauma-induced mechanical unloading significantly decreased the bone density, made the distribution of Scl closer to the marrow on the anterior and medial side, and increased the Scl expression only on the lateral side. TEM findings showed that only the expression of Scl in canaliculi was increased by mechanical unloading.

Conclusion: Our results showed that even short-term mechanical unloading is enough to decrease bone density, and mechanical unloading not only regulated the Scl expression but also changed the Scl distribution in both the osteocyte network and subcellular structures.

Introduction

Mechanical unloading (reduced mechanical stress on the bone) induces significant bone loss, as evidenced by disuse osteoporosis, which is a critical issue for bedridden patients and astronauts [1, 2]. Mechanical loading is a crucial factor controlling bone mass [2].

Osteocytes are thought to be the major bone cell type responsible for sensing mechanical loading and orchestrating signals of bone resorption and formation [1, 3]. Osteocytes are former osteoblasts that become surrounded by unmineralized matrix during bone formation; they make up 90%-95% of the adult bone cell population [4]. They form a three-dimensional (3D) structural network and are extensively coupled to each other via their slender cell process [5, 6]. Osteocytes are proposed to play a significant role in bone mechanosensation based on their location and typical morphology [7, 8].

Sclerostin (Scl, SOST) is a protein encoded by the *SOST* gene that is primarily secreted by osteocytes and is known to be a negative regulator of the bone mass [8, 9]. Scl binds to low-density lipoprotein receptor-related proteins (LRP) 5, LRP 6, and frizzled receptors to antagonize Wnt/βcatenin signaling [10–13]. Wnt/βcatenin signaling is crucial for osteoblast differentiation. Therefore, Scl inhibits bone formation [14–20].

SOST knockout mice have an extremely high bone mass, similar to that found in cases of sclerosis and Van Buchem's disease, which are genetic diseases in humans resulting from the complete absence of active sclerostin caused by a mutation in *SOST* [21, 22]. Furthermore, Scl appears to be central to the response of bone to mechanical loading [20]. The Scl expression increases with mechanical unloading and decreases with mechanical loading [16, 20, 23, 24]. Furthermore, in mechanically loaded cortical bone, Scl is localized inside the osteocyte cell body. In contrast, in unloaded cortical bone, Scl is distributed not only inside the osteocyte cell body but also in the lacuno-canalicular network [16]. The distinct local expression pattern of Scl by osteocytes may contribute to the site-specific activity of bone turnover, thus ultimately regulating local modeling and remodeling [17]. However, the mechanism underlying this change is unclear,

72   as mature osteocytes mainly produce Scl [25, 26].

73   Mature osteocytes are characterized by few cytoplasmic organelles [27]. Therefore, it might

74   be difficult to attribute the significant change in the Scl distribution only to changes in the Scl

75   production. Furthermore, the details concerning the Scl distribution in osteocytes by mechanical

76   unloading remain unclear.

77   Age-related cortical thinning is mainly caused by endocortical resorption [28]. In addition,

78   previous studies [29, 30] have reported a higher response to loading at the endocortical surface

79   than the periosteal surface. However, a study including a finite element analysis showed that the

80   greater response to loading at the endocortical surface is not due to the endocortical surface

81   receiving more mechanical stimuli [31].

82   In the present study, we hypothesized that mechanical unloading induced changes in not only

83   the Scl expression but also the distribution of Scl. To observe the inter- and sub-cellular Scl

84   distribution in the osteocytic lacuno-canalicular system, we performed immunogold staining with

85   transmission electron microscopy (TEM). However, the region that we were able to observe by

86   TEM was quite small. We had to observe a wide area of the bone tissue in order to detect the

87   region that was sensitive to mechanical loading. Therefore, before performing TEM observation,

88   we conducted immunofluorescent staining and observed the bone tissue with a confocal laser

89   scanning microscopy system. After identifying the regions showing significant mechanical

90   unloading-induced changes in the Scl expression, we observed the osteocytes in those regions by

91   TEM. We then analyzed the precise Scl distribution in osteocytes under loading and unloading

92   conditions.

Materials and Methods

Animals

All animal experimental protocols were conducted in accordance with the guidelines of the animal care and use committee of Okayama University (OKU-2016367). Ten male, 12-week old Sprague Dawley rats were purchased from Japan SLC (Shizuoka, Japan). Rats were kept at 21-24 ℃ and given free access to water and food.

Experimental design

After isoflurane-induced anesthesia, as shown in **Figure 1A upper panel**, rats received an approximately 2-cm incision on the right thigh, and the fascia was incised. The sciatic nerve was then dissected out and excised to about 5 mm. Also, an approximately 2-cm incision was made at the right inguinal region (**Fig. 1A lower panel**), and the femoral nerve was dissected out and excised to about 5 mm (NX group: unloading condition). On the opposite side, the sciatic and femoral nerves were exposed but were not excised, as a sham operation (sham group: loading condition). Since the sciatic nerve controls the muscle of the flexor of the posterior region of the femur and the femoral nerve controls the muscle of the anterior region, excising both the sciatic and femoral nerves fully immobilized the target leg.

Sample preparation

Seven days after surgery, anesthetized rats were perfused and fixed with 4% paraformaldehyde in phosphate-buffered saline (PFA/PBS), and their right and left femurs were dissected. Femurs were immersed in 17% EDTA (OSTEOSOFT; Merck Millipore, Burlington, MA, USA) for 28 days at 4 ℃ for demineralization. Samples for immunohistochemistry were embedded in paraffin and cut into 7-µm-thick coronal sections. Serial sections were collected onto silane-coated slides. Samples for immunogold staining were embedded in LR-White (London Resin Company Ltd., London, UK) and cut into 90-nm-thick sections.

117 ***Bone density measurement by peripheral quantitative computed tomography (pQCT)***

118 The bone density of the femoral diaphysis and distal metaphysis (3.0 mm mesial from growth

119 plate) was analyzed by pQCT (XCT Research SA+; Stratec Medizintechnik GmbH, Pforzheim,

120 Germany). Femurs were scanned with 0.46-mm slice thickness and 0.12-mm voxel size. Analyses

121 were performed using the XCT 6.20 software program (Stratec Medizintechnik GmbH). The

122 femoral contour was detected by Contour mode 2. The following definitions were used: cancellous

123 bone corresponded to regions with 35% of the total cross-sectional area having a relatively low

124 density (defined by Peel mode 20), while cortical bone corresponded to regions with a density of

125 over 690 mg/cm^3 (defined by Cortical mode 1). The bone density measurement by pQCT was

126 performed by Kureha analysis center Co, Ltd., Tokyo, Japan.

127 ***Immunofluorescence studies of paraffin-embedded sections***

128 Sections of femoral diaphysis were blocked in universal blocking reagent (Nacalai Tesque, Kyoto,

129 Japan) before incubation with a goat polyclonal antibody against *SOST*/Sclerostin (AF1589; R&D

130 Systems, Minneapolis, MN, USA; 1:200) at 4 ℃ overnight, followed by a species-matched Alexa

131 Fluor 488-conjugated (Molecular Probes, Eugene, OR, USA; 1:200) secondary antibody at room

132 temperature for 1-h. For negative controls, nonimmunized goat IgG was used as a substitute for

133 the primary antibody. Nuclei were stained using 4'6-diamidino-2-phenylindole (DAPI; Sigma

134 Aldrich, St. Louis, MO, USA). After rinsing the samples, the samples were embedded in

135 fluorescence mounting medium (Dako, Carpinteria, CA, USA).

136 ***Confocal fluorescence tiling imaging***

137 Confocal tiling imaging for the immunostained femur sections was performed with an LSM 780

138 confocal laser scanning microscopy system (CarlZeiss, Oberkochen, Germany) and C-

139 Apochromat (63x/1.20 W Korr M27) and Plan-Apochromat (20x/0.8 M27) at Central Research

140 Laboratory, Okayama University Medical School, Japan. Two laser lines of 405 nm and 488 nm

141 were used. Images of 1.25 μm/pixel and 12-bit color depth were acquired.

142   *Quantification of Scl distribution on immunofluorescence images*

143   We then divided the above-mentioned stitched fluorescence image into anterior, posterior, medial,

144   and lateral regions (**Fig. 1B**). The fluorescence intensity was measured from the bone marrow side

145   to the periosteum side of the cortical bone with a width of 400 μm. We constructed mean profiles

146   by averaging the fluorescence intensity (**Fig. 1C-1**) that perpendicular to the arrow indicated in

147   each ROI region in **Fig. 1B**. Those profiles were then converted to a curve of the distribution

148   tendency (**Fig. 1C-1**) using an empirical mode decomposition (EMD) method [32]. EMD was

149   performed using "EMD" and "pracma" software packages in the R program language. The

150   resource R script for processing our data in this study can be checked in the author's GitHub

151   (https://github.com/wong-ziyi/Eccentricity). As shown in **Fig. 1C**, this EMD method could

152   identify the peak of the Scl distribution tendency and avoid the influence of outliers in the original

153   profiles of fluorescence intensity (**Fig. 1C-1**). Eccentricity was defined as Eccentricity = (2S −

154   L)/L, where S is the location of the peak of the distribution tendency, and L is the total length of

155   the ROI region. Since we set the boundary of the marrow as 0, positive eccentricity indicates that

156   the peak of the Scl distribution tendency is closer to the marrow side, while negative eccentricity

157   indicates that it is closer to the periosteal side. The number of cells with positive staining for Scl

158   was analyzed using the "Analyze Particles" function after auto-thresholding with the default

159   method using the ImageJ/Fiji software program (US National Institute of Health, Bethesda, MD,

160   USA).

161   *3D image construction*

162   The 3D immunofluorescence images were constructed from the z-series of CLS images using the

163   IMARIS software program (Bitplane, Zurich, Switzerland) as described previously [17, 33].

164   *Immunoelectron microscopy and quantification of the Scl distribution*

165   Ultrathin sections were etched in 0.05 M glutaraldehyde and 6 M urea for 10 minutes. After

166   rinsing, the sections were blocked in 1% bovine serum albumin and 10% rabbit serum for 15

167   minutes. Sections were incubated with goat polyclonal antibody against *SOST*/Sclerostin

168   (AF1589; R&D Systems) (1:100) at 4 ℃ overnight, followed by a rabbit anti-goat IgG conjugated

169   with 10-nm colloidal gold (EMARG10; BBI Solutions, Crumlin, UK) (1:50) secondary antibody

170   at room temperature for 2-h. After washing, the samples were fixed with 1% glutaraldehyde and

171   then observed and photographed via TEM (H7650; HITACHI, Tokyo, Japan) at Central Research

172   Laboratory, Okayama University Medical School. The Scl distribution was evaluated by

173   calculating the Scl density per unit area of the osteocyte cell body, lacuna, and canaliculi. Acquired

174   images were analyzed using the Image J software program.

175   ***Statistical analyses***

176   A paired *t*-test was applied to determine the significant difference between the sham and NX group.

177   We performed two-way analyses of variance (ANOVAs) with paired samples to test whether or

178   not an interaction effect between NX and position existed. The followed Fisher's Least Significant

179   Difference with a single pooled variance was performed for multiple comparisons among different

180   regions (anterior, posterior, medial, and lateral regions for immunostaining images and the cell

181   body, lacuna, and canaliculi for TEM images) in each treatment group (sham or NX).

182   In the above statistical analyses, measurements at $P<0.05$ were considered statistically

183   significant. All data are expressed as the mean ± standard deviation (SD).

184   All of these statistical analyses were performed using either the GraphPad Prism 6 software

185   program (GraphPad Software, Inc., San Diego, CA, USA) or the R software program

186   (https://www.R-project.org/).

Results

The bone density is decreased in the distal metaphysis of the femur under unloading conditions

After fully immobilizing (please check the rat's ambulatory in **Online Resource 1**) the right leg seven days, pQCT showed that, in the distal metaphysis of the femur, the total bone density, cortical bone density, and cancellous bone density were decreased in the NX group (**Fig. 2A**). In contrast, no significant changes in the femoral diaphysis were noted between the sham and NX groups (**Fig. 2B**).

Mechanical unloading significantly changed the Scl expression in the lateral and medial regions of the femoral diaphysis.

According to the two-way ANOVA, dissection of the femoral nerve (NX) contributed to 11.74% and 20.18% of the total variance for the NX-induced changes in the Scl distribution (**Fig. 2C upper panel**) and expression (**Fig. 2C lower panel**), respectively. The upper panel of **Fig. 2C** showed that NX-induced unloading brought the distribution of Scl significantly closer to the marrow on the anterior and medial side, while the expression of Scl was significantly increased by NX-induced unloading only on the lateral side (**Fig. 2C lower panel**). No interaction effect was observed in the Scl distribution (**Fig. 2C upper panel**) or expression (**Fig. 2C lower panel**).

Interestingly, the Scl distribution differed among the four regions in the Sham group. The Scl distribution in the anterior and medial regions was closer to the periosteal side than that in the posterior and lateral regions (**Fig. 2C upper panel**). However, there were no significant differences in the eccentricity in any region in the NX group (**Fig. 2C upper panel**). These results imply that the lateral region is sensitive to mechanical loading.

The location closest to the periosteum in the lateral region of the femoral diaphysis is where the Scl distribution is likely to change due to mechanical unloading

As shown in **Fig. 3A**, we divided the lateral region into four equal parts (I, II, III, and IV) to identify the region most drastically changed in response to unloading for further TEM observation.

212 We counted the number of Scl-positive osteocytes and calculated the ratio of Scl-positive

213 osteocytes to total osteocytes in each of the four parts (**Fig. 3C**). There were significantly more

214 Scl-positive osteocytes in part Ⅳ of the NX group than in the Sham group (**Fig. 3C**). When

215 checking 3D images of Scl immunofluorescence, we noted dynamic changes in the Scl

216 distribution in the osteocytic lacuna-canalicular system in part Ⅳ (**Fig. 3D**), which was further

217 confirmed by TEM observations.

218 ***The mechanical unloading increased the Scl expression only in the canaliculi***

219 Since most changes in the Scl expression were observed in part Ⅳ of the lateral region, as

220 mentioned above, the subsequent TEM observation was focused on this specific site. Using

221 immunoelectron microscopy, we observed the Scl distribution inside the osteocyte cell body,

222 lacuna, and canaliculi (**Fig. 4A**). We counted the Scl molecules labeled by 10-nm colloidal gold

223 and measured the density per square micrometer in the osteocyte cell body, lacuna, and canaliculi

224 (**Fig. 4B upper panel**). The Scl density was significantly higher in the canaliculi of the NX group

225 than in the Sham group. We also analyzed the differences in the Scl density in canaliculi, lacuna,

226 and cell body between the Sham and NX groups. In the Sham group, the Scl density was higher

227 in the canaliculi than in the lacuna, while no significant difference in the Scl density was noted

228 between the cell body and lacuna or canaliculi (**Fig. 4B upper panel**). In the NX group, the Scl

229 density was significantly higher in the canaliculi than in the cell body or lacuna, with no significant

230 difference noted between the cell body and lacuna. Although a two-way ANOVA showed a

231 significant contribution of both the position and NX, there was no significant interaction effect

232 between the position and NX (**Fig. 4B upper panel**).

233   Intriguingly, NX-induced mechanical unloading did not induce any significant area changes

234 in the cell body, lacuna, or canaliculi. Indeed, a two-way ANOVA analysis also showed that NX

235 had no significant contribution to the area difference (**Fig. 4B lower panel**). Notably, there was

236 no marked in the density of Scl between cell bodies and their respective lacunae.

Discussion

The present study aimed to analyze the Scl distribution in osteocytes under loading and unloading conditions. Furthermore, we considered whether or not Scl transportation was related to changes in its distribution.

We prepared an animal model of disuse osteoporosis. First, using confocal laser scanning microscopy, we observed a wide region of the femur section immunolabeled with an antibody against SOST/sclerostin. We then detected the regions where the change in the Scl distribution was sensitive to mechanical loading. Second, in those same regions, we labeled anti-Scl antibody with 10-nm gold colloid and observed osteocytes using TEM. In contrast to the rodent models of disuse osteoporosis that have been generated via tail suspension and neurectomy of the lower hind limb [16, 19], in the present study, we performed unilateral neurectomy to obtain mechanically loaded and unloaded cortical bone in the same rat. The results of pQCT showed that the density of cortical bone and cancellous bone in the distal metaphysis of the femur was significantly decreased under unloading conditions, as previously described [34]. Therefore, our study of mechanical unloading in an animal model was well controlled.

Osteocytes reportedly tend to align themselves in the direction of the principle mechanical loading direction [35, 36]. The difference in the orientation of the osteocyte cell bodies in cancellous bone of the femur between loaded and unloaded conditions is not as significant as those detected in the cortical bone of the femoral midshaft. A previous computer simulation study reported that the external forces exerted on the cortical bone were greater than those exerted on cancellous bone [37]. Femoral diaphysis is suitable for observing the loading-related strain distribution [16]. We therefore performed immunofluorescence staining in coronal sections of the femoral diaphysis. Different positions (anterior, posterior, lateral, or medial) did not contribute significantly to the variance of the Scl distribution, and no interaction effect was observed on a two-way ANOVA (**Fig. 2C lower panel**), which suggested that the changes in the peak of the Scl distribution tendency were mainly due to the NX-induced mechanical unloading. In the lateral

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263 and anterior regions, the peak of the Scl distribution tendency shifted to the region adjacent to the

264 marrow under NX-induced mechanical unloading (**Fig. 2C upper panel**). However, only the

265 lateral region showed significant changes in the fluorescence intensity between the Sham and NX

266 groups, with no such changes noted in the anterior, posterior, or medial regions (**Fig. 2C lower**

267 **panel**).

268   We then divided the lateral region into four parts to analyze the Scl distribution in more detail

269 by identifying Scl-expressing cells (**Fig. 3A**). In the lateral region of the NX group, the Scl-

270 expressing cell count was higher than in the Sham group in the area closest to the periosteum (**Fig.**

271 **3C**). These results imply that loading induced the most marked changes in the Scl distribution in

272 the periosteal side of the lateral regions. We then constructed 3D images to analyze the Scl

273 distribution around osteocytes, with the images showing that Scl was localized to a narrow area

274 under loading conditions but spread more broadly under unloading conditions.

275   Next, in the same regions, we analyzed the Scl distribution inside the osteocyte cell body,

276 lacuna, and canaliculi under loading and unloading conditions via immunogold staining with TEM.

277 There were no significant differences in the Scl density inside the cell body and lacuna between

278 the NX and Sham groups (**Fig. 4B upper panel**). However, the Scl density inside the canaliculi

279 was significantly higher in the NX group than in the Sham group (**Fig. 4B upper panel**). In the

280 NX group, the Scl density was significantly higher inside the canaliculi than inside the osteocyte

281 cell body or lacuna (**Fig. 4B upper panel**). According to the two-way ANOVA, different positions

282 (cell body, lacuna, or canaliculi) contributed the most to the variance in the Scl expression.

283 However, no interaction effect was observed, which suggested that the mechanical unloading-

284 induced changes in the Scl expression were site-specific (**Fig. 4B upper panel**). Those findings

285 indicated that the Scl expression was denser in the canaliculi than at other sites (i.e., cell body and

286 lacuna), and the expression in the canaliculi was more sensitive to mechanical unloading than that

287 at other sites. Those findings are consistent to our previous report that the mechanosensitivity of

288 the cell processes was higher than that of the cell body in osteocyte [38].

289   As shown in the upper panel of **Fig. 2C**, Scl distributed more to the marrow side in the

290   anterior and medial regions of the NX group than the Sham group. In addition, the anterior and

291   medial regions were more sensitive to NX-induced mechanical unloading than posterior or lateral

292   regions since the peak of Scl distribution tendency was shifted from the periosteal side to the

293   marrow side (**Fig. 2C upper panel**). However, the overall expression of Scl indicated by the

294   fluorescence in **Fig. 2C lower panel** was unchanged. These findings suggested that Scl

295   distribution was more sensitive to mechanical stimuli than the Scl expression. In other words,

296   solute transport is more sensitive to mechanical stimuli-induced expression changes than Scl

297   synthesis. Consistent with our findings, based on the finite element analysis, a previous report

298   found that the endocortical surface showed a greater response to loading but received less

299   mechanical stimuli [31]. The changes in the Scl distribution among osteocytes observed in the

300   present study may explain this paradox [31]. Indeed, our findings suggest a possible "energy"-

301   saving strategy for bone in response to mechanical stimuli, since the relative concentration of Scl

302   was increased near the marrow side (**Fig. 2C upper panel**). However, the overall concentration

303   may not have been changed (**Fig. 2C lower panel**) under NX-induced mechanical unloading.

304   Our TEM results showed that the Scl density in the osteocyte cell body and lacuna did not

305   differ markedly between the Sham and NX groups (**Fig. 4**), which showed a relatively high

306   concentration of sclerostin in the lacuno-canalicular system outside of osteocytes. Our previous

307   study [39] suggested that the fluid flow profile in the lacuno-canalicular system is determined by

308   the morphology of the canalicular wall. However, the morphology of the lacuno-canalicular

309   system is susceptible to being changed by bone matrix deformation under different mechanical

310   loading environments. Thus, the relatively high concentration of sclerostin outside of the

311   osteocytes facilitated the re-distribution of Scl in the lacuno-canalicular system when the

312   mechanical loading environment changed. Since transportation of Scl outside of the osteocytes in

313   the lacuno-canaliculi occurs more rapidly than changing the Scl expression, the above may be a

314   potential mechanism rendering Scl distribution more sensitive to mechanical stimuli than the Scl

315 expression.

316   Solute transport through the lacuno-canalicular system occurs by both diffusion and

317 convection [40]. Diffusion depends on solute concentration gradients, while convection depends

318 on movement through the pericellular space. Since the fluid is incompressible, the deformation of

319 bone under mechanical loading forces it to flow (like squeezing a sponge), as demonstrated by

320 Piekarski and Munro [41]. Forces that induce fluid flow are mainly generated by muscular actions

321 and reactions due to physical activity [41, 42]. Solute transport in the lacuno-canalicular system

322 is dependent on the size of the molecules, with small solutes under 1 kDa or roughly 1 nm in

323 diameter (e.g., glucose and ATP) being easily transported by diffusion, while larger solutes in the

324 range of 10-70 kDa or up to 6 nm in diameter (e.g., Scl, *RANKL* and parathyroid hormone) require

325 convective transportation [43–47].

326 **Limitations**

327   In the present study, the area of the cell body, lacuna, and canaliculi did not show any marked

328 differences between the Sham and NX group, which suggested that we did not observe any

329 osteocytic osteolysis that was recently confirmed in lactation [48], space flight [49], and hindlimb

330 suspension [50] mouse models. The treatment duration (seven days) in this study was shorter than

331 that in those previous reports (two to three weeks) that observed osteocytic osteolysis, so

332 osteocytic osteolysis may not be an early response to mechanical unloading. Alternatively, this

333 discrepancy may have been due to the femoral and sciatic nerve dissection performed in this study,

334 as emerging evidence has indicated that innervation is important for bone remodeling [51].

335 However, the underlying mechanism will need to be explored in further investigations.

336 **Summary**

337   Our results showed that even a short-term (seven days) mechanical unloading was able to

338 decrease the bone density (**Fig. 2A**). However, the Scl distribution showed more significant

339 changes than the Scl expression (**Fig. 2C** and **Fig. 3**), possibly due to a relative high concentration

340 of Scl outside of osteocytes in lacuno-canaliculi (**Fig. 4**). Interestingly, the short-term mechanical

14

341   unloading in the present study increased the Scl expression only in the canaliculi (**Fig. 4B upper**

342   **panel**), and the unchanged area in subcellular structures indicated no osteocytic osteolysis (**Fig.**

343   **4B lower panel**).

344   The present findings suggested that, given the relatively high concentration of Scl outside of

345   osteocytes in the lacuno-canaliculi, changes in the Scl distribution in both the osteocyte network

346   and subcellular structures were early events in response to neurotrauma-induced mechanical

347   unloading. Given the findings of previous studies, early changes in the Scl distribution may play

348   a crucial role in the bone's response to mechanical stimuli, which should be further explored in

349   future investigations.

350   **Author Contributions**

351   H.K., R.O., T.I., and Z.W. designed the study. R.O., N.O., T.I., and Y.I. conducted the study. Z.W.

352   tested the existing code components, programmed script for data processing, and drew the sketch.

353   R.O. and Z.W. processed, analyzed, and visualized the data and wrote the manuscript. R.O., Z.W.,

354   T.I., Y.I., and H.K. interpreted the data and approved the final version of the manuscript. H.K. is

355   responsible for the integrity of the data analysis. R.O and Z.W. contributed equally to this work.

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515   **Figure legends**

516   **Fig. 1** Experimental design and quantification method of immunofluorescence images.

517   (**A**). A schematic diagram of the excision of the sciatic and femoral nerves. LN, Lumber Nerve.

518   (**B**). A schematic diagram of the scanning position for pQCT and the coronal serial sections of

519   the midshaft of the sham and NX groups. We divided the section into anterior, posterior, medial,

520   and lateral regions (see red dotted lines). The asterisks indicate bone marrow. The spatial

521   immunofluorescence intensity was measured according to the direction indicated by the red

522   arrow. (**C**). An example of a profile analysis. (1) The fluorescence intensity profiles of the

523   region from the bone marrow side to the periosteum side of the cortical bone (400 μm wide).

524   The vertical axis of the graph indicates the intensity, and the horizontal axis indicates the

525   distance from the bone marrow side of cortical bone. "0" on the horizontal axis indicates the

526   border between the cortical bone and periosteum. (2) The graph with one maximum value

527   converted from the profile using empirical mode decomposition. The distance from the center

528   of the cortical bone to the maximum value was measured. We divided the value by the distance

529   from the bone marrow to the periosteum side and defined it as the eccentricity. Using the

530   eccentricity as an index, we evaluated the Scl distribution

531   **Fig. 2** The findings from pQCT and immunofluorescence showing the NX-induced changes in the

532   femoral bone density and sclerostin distribution, respectively.

533   The total bone density, cortical bone density, and cancellous bone density of the femoral (**A**)

534   distal epiphysis and (**B**) femoral midshaft were evaluated by pQCT. (**C**)**.** The eccentricity and

535   average fluorescence intensity of sclerostin on the anterior, posterior, lateral, and medial sides

536   were quantified in the Sham and NX groups. The data of (**A**) and (**B**) and the lower panel of (**C**)

537   are expressed as the mean ± SD (n=5 for pQCT and n=4 for immunofluorescence imaging); the

538   data of the eccentricity are presented by Tukey's box-and-whisker plot. ANOVA, analysis of

539   variance; ※ Paired *t*-test; *, uncorrected Fisher's LSD test with a single pooled variance

540   followed by a two-way analysis of variance with the paired sample. ※ *, $p < 0.05$; ※※ **, p

541   < 0.01.

542   **Fig. 3** The number of sclerostin-positive osteocytes in the lateral region was evaluated.

543   (**A**). Confocal tiling images of the lateral region. The sections were immunostained for Scl

544   (green), and nuclei were stained with DAPI (blue). The white dashed line indicates the border

545   between the marrow and cortical bone. The alternate long- and short-dash line indicates the

546   border between the periosteal and cortical bone. The area from the marrow side to the periosteal

547   side of the cortical bone was divided into four regions: I, II, III, and IV. Scale bar=100 μm. (**B**)**.**

548   Magnified images of regions I, II, III, and IV. Scale bar =20 μm. (**C**). The ratio of Scl-positive

549   osteocytes to total osteocytes (nuclei stained with DAPI) in regions Ⅰ, Ⅱ, Ⅲ, and Ⅳ. The data

550   are expressed as the mean ± SD (n=4). The asterisks indicate significant differences from the

551   Sham group (*, $p < 0.05$: paired *t*-test). (**D**). Three-dimensional immunofluorescence images of

552   osteocytes in regions Ⅳ constructed using the IMARIS software program. Three-dimensional

553   fluorescence images (upper figures) were constructed using the IMARIS software program.

554   Surface rendering images corresponding to lower figures are shown. Scl and the nuclei are

555   indicated in green and blue, respectively. Scale bar=10 μm.

556   **Fig. 4** The Scl distribution inside the osteocyte cell body, lacuna, and canaliculi was evaluated by

557   immunoelectron microscopy.

558   (**A**). The analysis of Scl labeled by 10-nm gold colloid using TEM in the lateral region. (a, b)

559   TEM images. The red single-dot chain line indicates the border between the osteocyte cell body

560   and lacuna. The blue dotted chain line indicates the border between the lacuna and bone matrix.

561   The yellow double-dot chain line indicates the border between the canaliculi and the bone

562   matrix. Scale bar=3 μm. (c-f) Magnified images of the white square border. Scale bar=300 nm.

563   (**B**). Scl density per unit area (upper panel) and the area (lower panel) of the osteocyte cell body,

564   lacuna, and canaliculi. The data are expressed as the mean ± SD (n=4); *, uncorrected Fisher's

565   LSD test with a single pooled variance followed by a two-way analysis of variance with the

566   paired sample; **,** $p < 0.05$; **, $p < 0.01$.

567   **Online Resource 1.** A video to show that the entire target leg was fully immobilized.
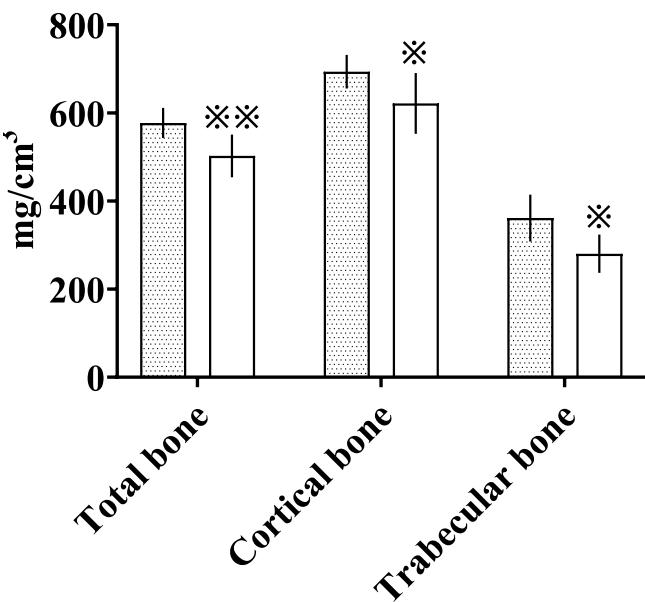
568   In this video, the rat was walking with a slow dragging motion without lifting the right foot. We

569   shot this video one day after the excision of the sciatic and femoral nerves.
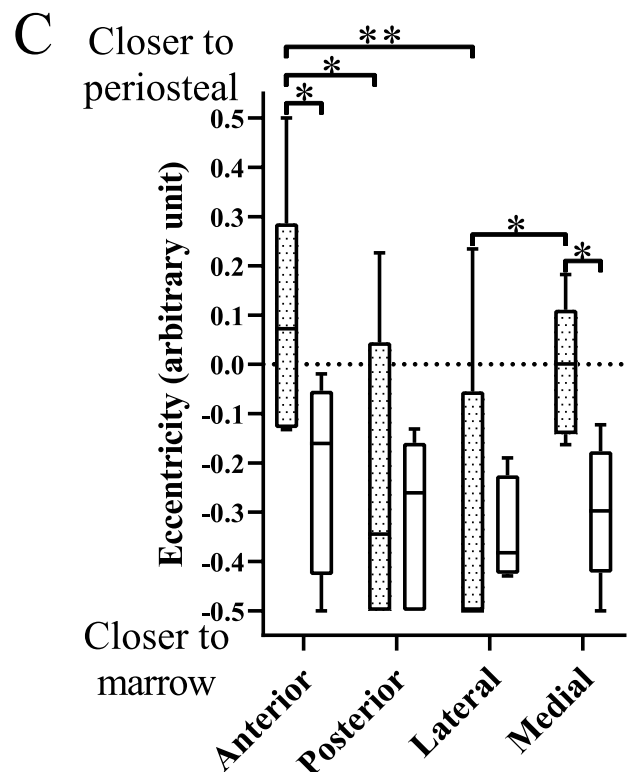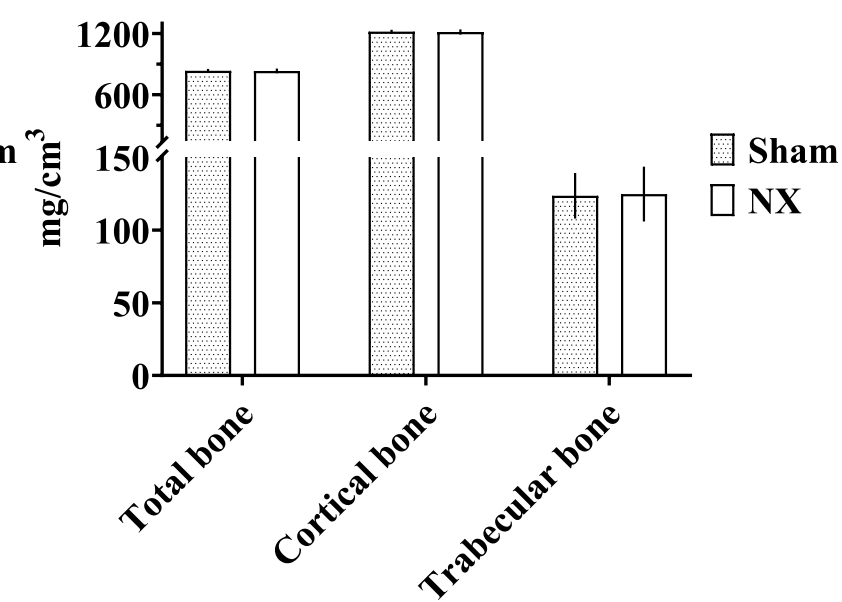
Figure1
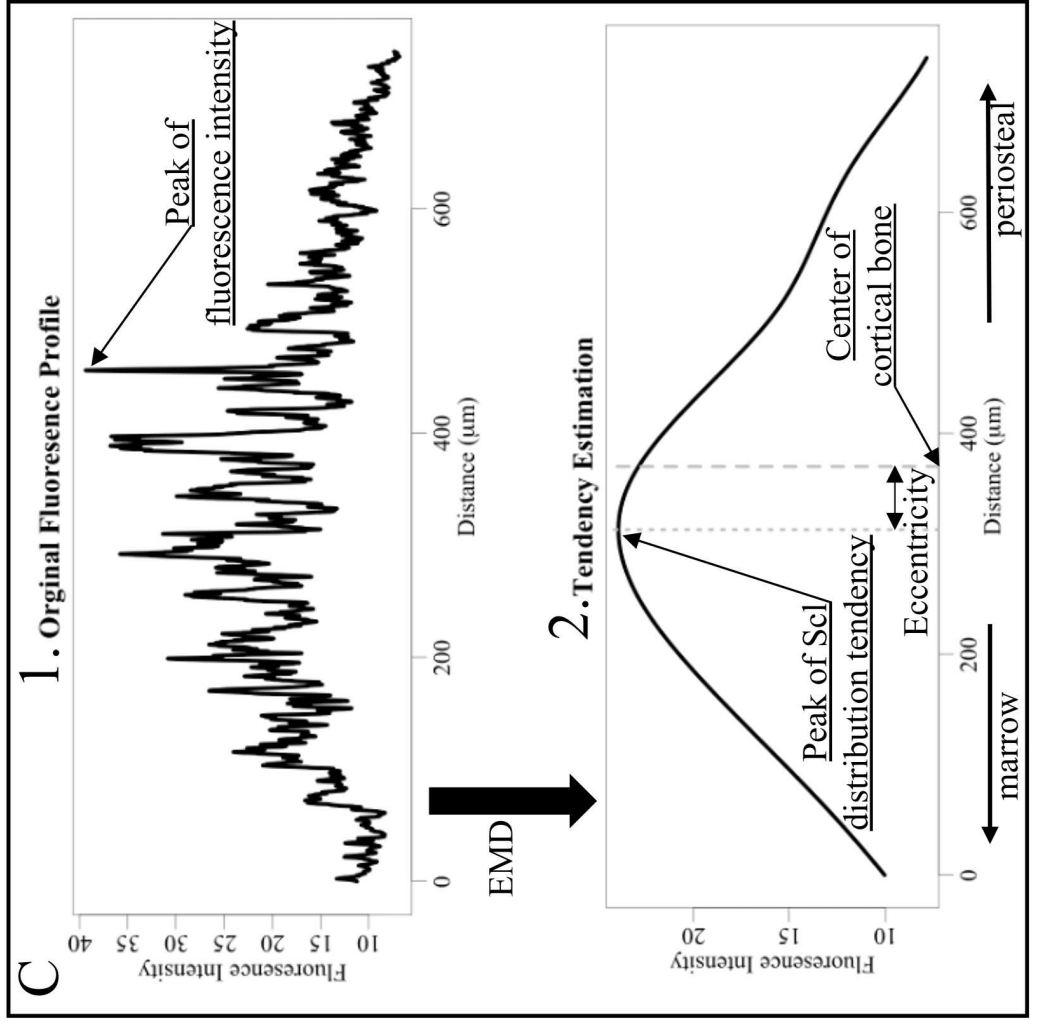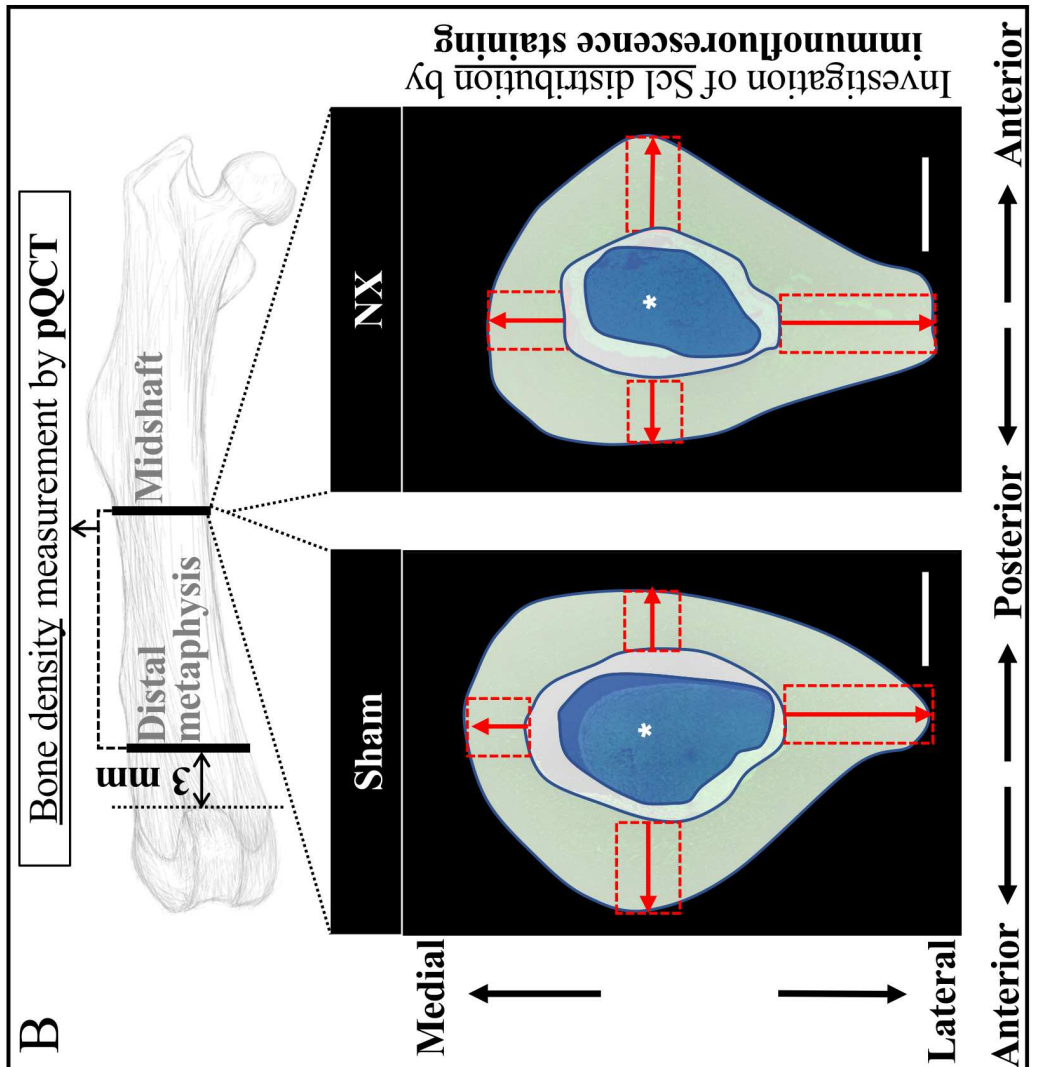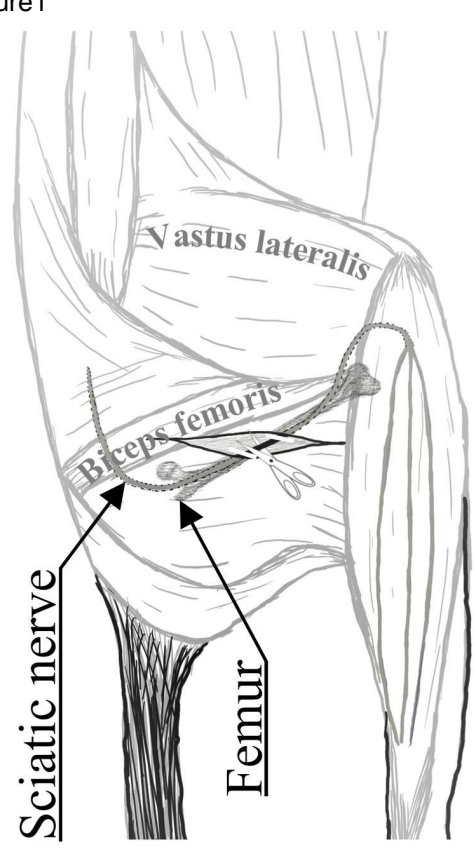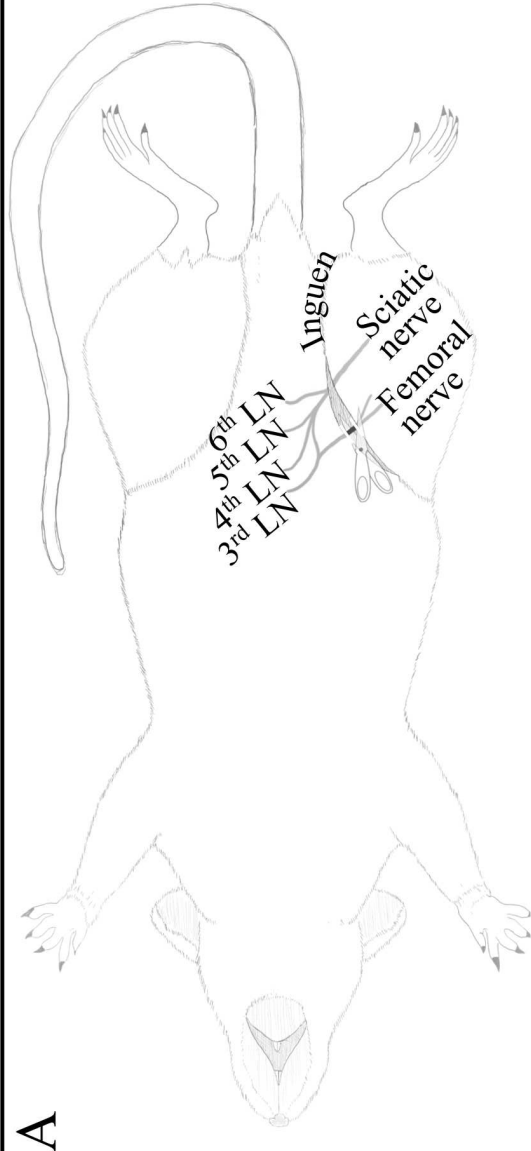
Figure2

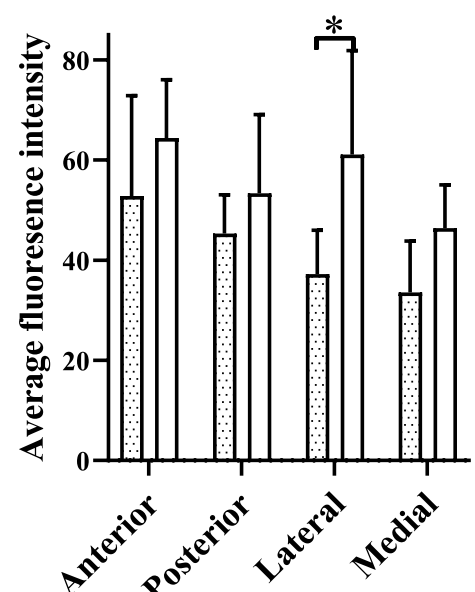**A** Bone density of distal metaphysis

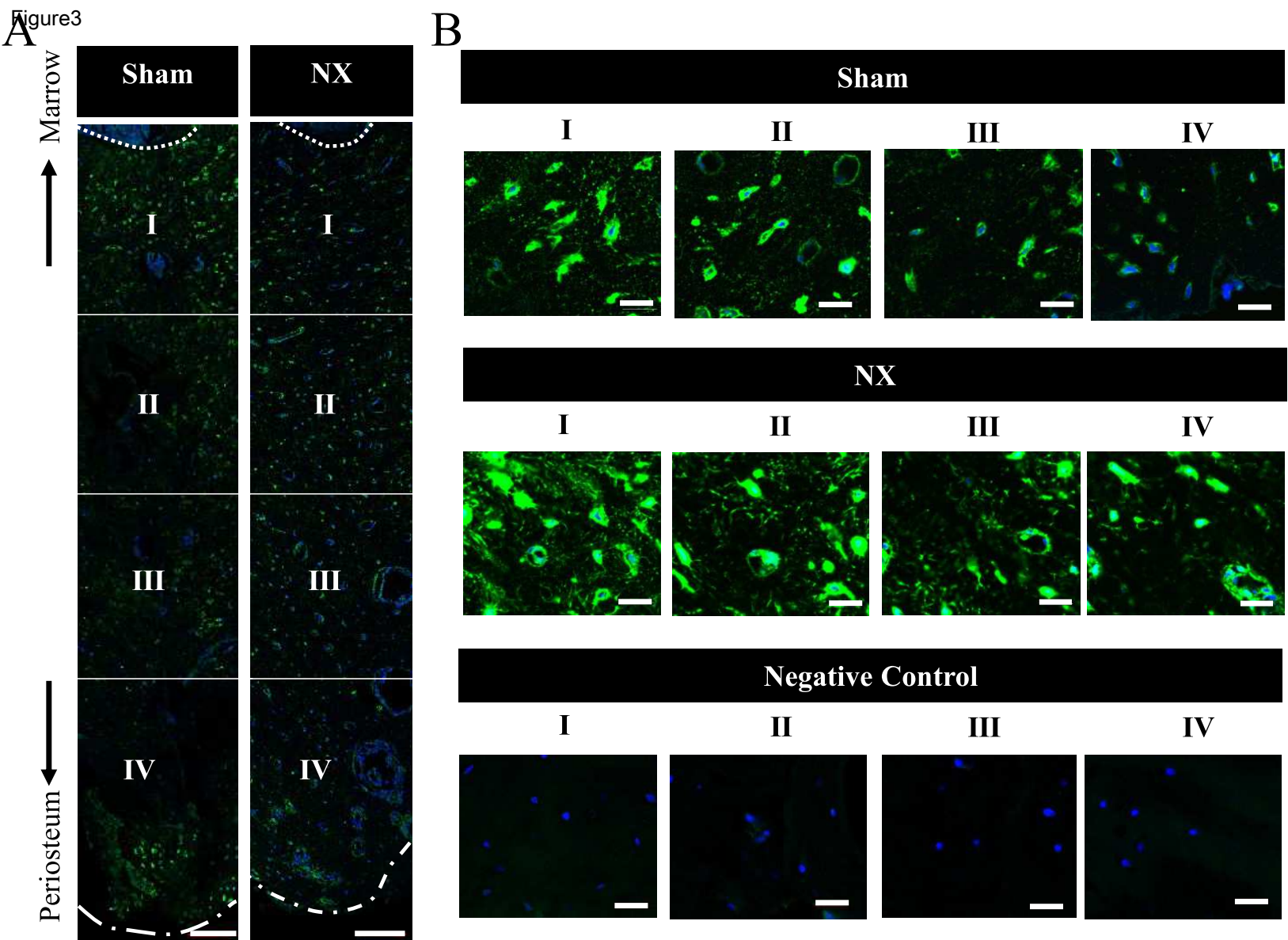**B** Bone density of diaphysis

C

Two-way ANOVA

Source of Variation	Percentage of total variation (%)	P value
Interaction	6.964	0.3398
Position	17.31	0.0508
NX	11.74	**0.0212**

Two-way ANOVA

Source of Variation	Percentage of total variation (%)	P value
Interaction	3.57	0.6939
Position	17.65	0.0918
NX	20.18	**0.0083**

Figure3

Figure4

A

| Sham | NX |

B

Two-way ANOVA

Source of Variation	Percentage of total variation (%)	P value
Interaction	6.07	0.0900
Position	12.66	**0.0110**
NX	9.03	**0.0008**

Two-way ANOVA

Source of Variation	Percentage of total variation (%)	P value
Interaction	0.04	0.9620
Position	38.45	**<0.0001**
NX	1.24	0.3099