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Influence of Mechanical Force on Bone Matrix Proteins in Ovariectomised Mice and Osteoblast-like MC3T3-E1 Cells

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Abstract. *Aim: To investigate the effect of mechanical stress on periostin and semaphorin-3A expression in a murine model of postmenopausal osteoporosis and in osteoblast-like MC3T3-E1 cells. Materials and Methods: Female mice were divided into three groups and treated with a sham operation, ovariectomy (OVX) or OVX plus treadmill training (OVX+Run). After 10 weeks, tibias were used for histological analysis. MC3T3-E1 cells were burdened by mechanical stress using a centrifuge or were treated with periostin, and the production of biologically-active semaphorin-3A was examined in vitro. Results: In OVX+Run group tibias, the number of tartrate-resistant acid phosphatase-positive osteoclasts was lower than in the OVX group, and the expression of periostin and semaphorin-3A was higher. In MC3T3-E1 cells, centrifugal stress significantly increased periostin and semaphorin-3A mRNA expression. Treatment with periostin increased the semaphorin-3A level. Conclusion: We speculate that mechanical load may increase periostin production in osteoblasts, and periostin may inhibit osteoclast differentiation by its effects on semaphorin-3A. Our results support the concept of a positive correlation between exercise and inhibition of osteoclasts in post-menopausal osteoporosis.*

Osteoporosis affects elderly men and women worldwide (1). The prevalence of osteoporosis in Europe in 2010 was 27.6 million people (2). The prevalence is predicted to rise exponentially with the accelerated expansion of the elderly population, especially in developing countries (3). Osteoporosis is a serious public health concern worldwide because of the morbidity and mortality associated with fragility fractures (4, 5).

Bone homeostasis is regulated by a remodelling process governed by three types of bone cells, osteoclasts that destroy and resorb bone tissue, osteoblasts that form new bone, and osteocytes (6). Bone turnover and bone remodelling are both tightly linked and regulated through the bone-forming and resorptive activities of osteoblasts and osteoclasts, respectively. Osteoporosis is a generalised skeletal disorder characterised by decreased bone mass and deteriorating bone architecture. Physical inactivity is a modifiable risk factor for osteoporosis (7). Preliminary studies have indicated a role for certain substances triggered by physical activity in the metabolism of bone tissue, *e.g.* prostaglandin E_2, oestrogen, osteoprotegerin and osteocalcin (8-10). Therefore, mechanical stress on bone plays an important role in the maintenance of bone homeostasis.

Periostin was originally isolated from a mouse osteoblast cell line (11, 12). Periostin is a component of the extracellular matrix expressed in collagen-rich connective tissues, such as bone (especially the periosteum), the periodontal ligament, the aorta and the heart valves (13, 14). Periostin is a secreted protein capable of interacting with various extracellular matrix components in addition to cell surface integrins. Periostin belongs to the matricellular protein family and is expressed after mechanical stress and fracture (15). Loss of periostin results in altered cancellous and cortical bone microarchitecture and lower bone mineral density (14). The role of periostin up-regulation by mechanical stress is probably related to bone tissue integrity. Additionally, several studies have analyzed the potential role of periostin in bone biology and suggest that periostin may be an important regulator of bone formation.

Semaphorin-3A was first found as an axon-guiding molecule and was recently shown to be secreted by osteoblasts and osteocytes (16). Administration of semaphorin-3A to mice increased bone volume and expedited bone regeneration by suppressing bone resorption and enhancing bone formation (16, 17). These reports suggest that semaphorin-3A may be a novel therapeutic agent for bone and joint diseases (18).

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Despite its initial identification in bone, investigations of periostin function in bone-related physiopathology are less abundant (19). Specifically, the influence of periostin on other bone metabolic factors, such as semaphorin-3A, when the bone is under mechanical stress still has not been completely verified. The aim of this study was to investigate the effects of periostin on semaphorin-3A expression in a murine model of postmenopausal osteoporosis and in osteoblast-like MC3T3-E1 cells.

Materials and Methods

Animals and exercise conditions. Specific pathogen-free, 7-week-old ICR female mice were purchased from Japan CLEA Co., Ltd. (Tokyo, Japan). Mice were maintained on a 12-h light/dark schedule (lights on at 8 a.m.) at 25±2˚C, humidity 50%±2% and provided with food and water *ad libitum*. After an acclimation period of 1 week, the mice were subjected to bilateral ovariectomy (OVX) or a sham operation *via* a dorsolateral incision after intraperitoneal administration of a combination anaesthetic [medetomidine hydrochloride, 0.3 mg/kg (Domitol; Nippon Zenyaku Kogyo Co., Ltd., Fukushima, Japan); midazolam, 4.0 mg/kg (Sandoz; Sandoz K.K. Tokyo, Japan) and butorphanol, 5.0 mg/kg (Vetorphale; Meiji Seika Pharma Co., Ltd., Tokyo, Japan)] (20, 21). For OVX, the ovaries were excised, oviducts were put back in their original position and the incisions were closed. The effect of ovariectomy was confirmed by measuring body and uterine weights at the time of the experiment (9, 10). After surgery, the animals were maintained under good conditions to allow them to recover. Mice were fed with a normal diet (CE-2; CLEA Japan, Tokyo, Japan) for 10 weeks from the day after surgery. After a rest period, the mice were divided into three groups of five mice/group: Sham, OVX and OVX+Run. The OVX+Run group underwent ovariectomy followed by physical training on a motorized treadmill for 10 weeks. The exercise training protocol was that described by Simões *et al*. (22): moderate exercise, initially for 10 min and then gradually increasing to 60 min per day, 4 days a week for 10 weeks. The speed was 5 m/min for the first 4 weeks and 10 m/min for the remaining weeks. After 10 weeks, all animals were sacrificed, the OVX+Run mice 24 h after the last exercise. The tibias were immediately excised for histological analysis. This study was approved by the Ethics Committee of Showa University for animal experiments (No. 05092).

Histochemical demonstration of tartrate-resistant acid phosphatase (TRAP) activity. The tibias were dissected free of surrounding tissue. Samples were then fixed in 4% neutral buffered formalin, decalcified in 10% EDTA–TBS, dehydrated and embedded in paraffin. Serial 4-μm-thick sections were then stained for TRAP activity (TRAP/ALP stain kit; Wako Pure Chemical Industries, Ltd., Tokyo, Japan). Briefly, deparaffinized and rehydrated tissue sections were incubated at room temperature in acetate-tartaric acid buffer (pH 5.0) for 20 min. Sections were then incubated at 37˚C for 15-20 min in freshly prepared acetate-tartaric acid buffer (pH 5.0) containing 0.5 mg/ml naphthol ASMX phosphate and 1.1 mg/ml Fast Red TR (TRAP substrate). After washing with distilled water, tissues were counterstained with aqueous haematoxylin and mounted with Vectamount (Vector Laboratories, Inc., Burlingame, CA, USA) (23).

Quantification of TRAP-positive cells. The number of osteoclasts appearing in digital images were captured with a BX53 System Microscope and a DP21 digital camera (Olympus Co., Tokyo, Japan) and counted within the primary *spongiosa* of the proximal tibia in a region of interest starting 1.0 mm proximal to the growth plate and extending 1.0 mm in length and 3.0 mm medially from the periosteum. The number of cells was determined in five tissue sections spanning 100 μm of tissue. Data are expressed as the mean number of cells per 1.0 mm of perimeter trabecular bone (23).

Immunohistological staining for detection of periostin and semaphorin-3A in tibia tissue. The tibia specimens were washed several times with saline to remove unwanted materials (*e.g*. blood and connective tissues), fixed in 4% paraformaldehyde–phosphate-buffered saline (PBS), decalcified in 10% EDTA-PBS, dehydrated, embedded in paraffin and cut into 4-μm-thick sections. A primary antibody for periostin (rabbit IgG, diluted 1:100; ab14041; Abcam Co., Ltd., Tokyo, Japan) was applied to the fixed cryosections and left overnight at 4˚C. To demonstrate endothelial integrity, a primary antibody for semaphorin-3A (rabbit IgG, diluted 1:200; bs-1121R; Bioss Inc., Woburn, MA, USA) was applied to fixed cryosections and incubated overnight at 4˚C. The sections were then washed with PBS and incubated for 1 h with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (ab6717, dilution 1:500; Abcam Co., Ltd) for periostin and Alexa Fluor® 555-conjugated donkey anti-rabbit IgG (A-31572, dilution 1:500; Life Technologies Japan Ltd., Tokyo, Japan) for semaphorin-3A. After rinsing in PBS, sections were coverslipped using VECTASHIELD mounting medium with 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Inc., Burlingame, CA, USA). Digital images were obtained using a confocal laser scanning microscope (A1si; Nikon Co., Ltd., Tokyo, Japan), and fluorescence was evaluated as above on at least four tibia sections per animal with NIT-Elements ver3.22 Analysis (Nikon Co., Ltd.).

Reagents and MC3T3-E1 cells. Periostin (recombinant mouse periostin/OSF-2, CF) was purchased from R&D systems, Inc. (Minneapolis, MN, USA) and dissolved in Dulbecco's modified Eagle's medium (DMEM; Sigma–Aldrich Corporation, St. Louis, MO, USA) supplemented with 10% heat-inactivated foetal calf serum (RPMI-FCS; Nihon Bio-Supply Center, Tokyo, Japan), sterilized by passing through 0.2-μm pore filters and stored at 4˚C until used. MC3T3-E1 cells, which are similar to osteoblasts, were purchased from DS Pharma Biomedical Co., Ltd. (Osaka, Japan) and resuspended in DMEM-FCS at 37˚C and 5% CO_2.

Mechanical stress on MC3T3-E1 cells. MC3T3-E1 cells were seeded at 5×10^5 cells/well in 24-well plates in triplicate. After 24 h, they were burdened by mechanical stress by use of a centrifuge (CF-7D; Hitachi Koki Co., Ltd., Tokyo, Japan) (24, 25). The cells were divided into control (no centrifugation), and groups centrifuged at 16 ×*g*) or 64 ×*g*). Centrifugation was carried out at 25˚C for 15 min. The culture supernatants were collected after 24 h, the medium was changed and then the cells were centrifuged again. After another 24 h, the culture supernatants were collected and the cells were recovered. The supernatants were stored at −80˚C until use for measurement of periostin and semaphorin-3A proteins by enzyme-linked immunosorbent assay (ELISA; see below). The MC3T3-E1 cells were collected for measurement of periostin and semaphorin-3A mRNA expression by real-time reverse transcription polymerase chain reaction (PCR; see below).

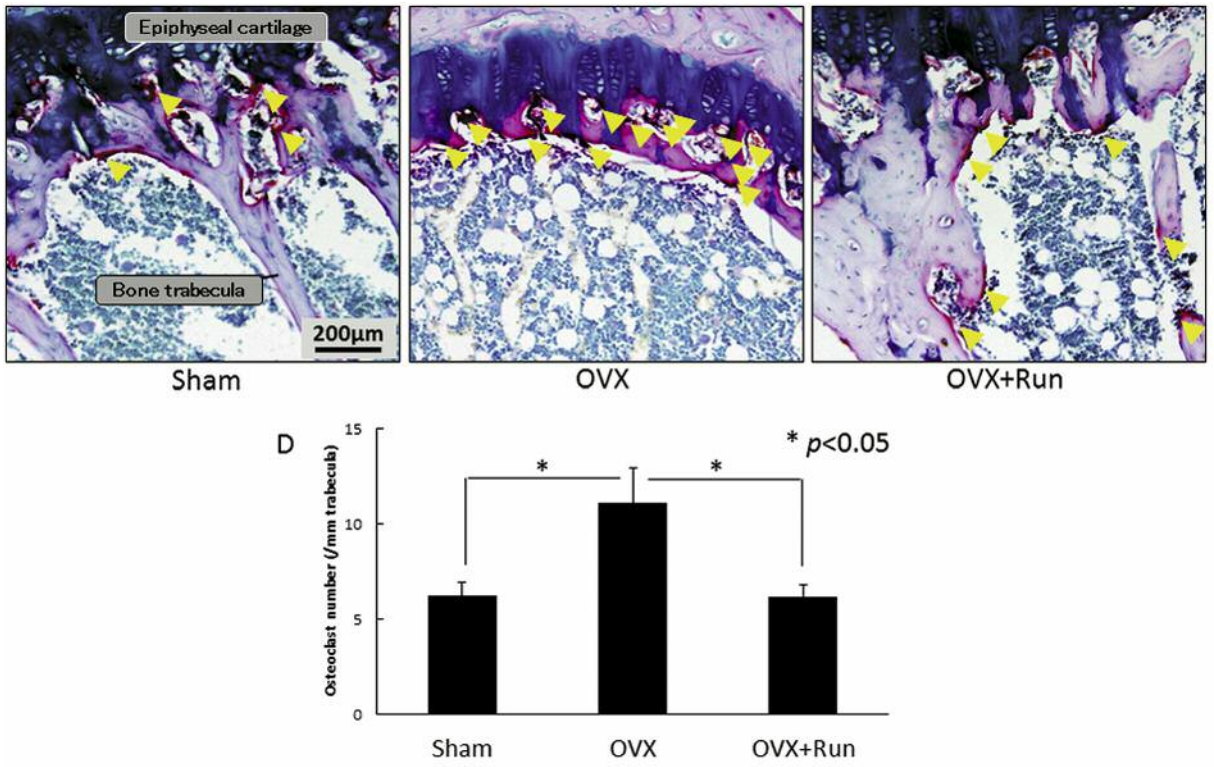

Figure 1. *Histological examination of tibia bone tissue. Mice were randomly divided into three groups of five mice/group: sham operation, ovariectomy (OVX) and ovariectomy plus treadmill training for 10 weeks (OVX+Run). The animals were sacrificed 24 h after the last exercise, and tibias were immediately excised for histological analysis and were stained with tartrate-resistant acid phosphatase (TRAP). Upper panel: The proximal tibias seen under optical microscopy. Sliced specimens were stained with TRAP and osteoclasts were counted (dark purple cells indicated by arrowheads). The blue areas are epiphyseal cartilage, and the pink areas are trabecular bone. Scale bars=200 μm. B: Number of TRAP-positive osteoclasts in trabecular bone. Data are expressed as mean cell number per mm of the trabecular bone. *Differences statistically significant at p<0.05. Error bars denote standard deviation.*

Effect of periostin on MC3T3-E1 cells. MC3T3-E1 cells were resuspended at a density of 5×10^5 cells/ml in DMEM-FCS and cultured with 0.1 or 1.0 ng/ml of periostin, using 24-well plates in triplicate. After 24 or 48 h, culture supernatants were collected and stored at −80˚C until use for measurement of semaphorin-3A protein by ELISA (see below). Additionally, MC3T3-E1 cells were collected and used for measurement of periostin and semaphorin-3A mRNA expression by PCR (see below).

Periostin and semaphorin-3A assays. Periostin and semaphorin-3A in culture supernatants were measured with commercially available ELISA test kits (MOSF20: R&D Systems, Inc., Minneapolis, MO, USA; CSB-EL020980MO: Cusabio Biotech Co., Ltd., Hubei Province, China) according to the manufacturer's recommendations. The minimum detectable level of the ELISA kits was 0.065 ng/ml for mouse periostin and 3.9 pg/ml for mouse semaphorin-3A. The absorbance at 450 nm was measured by a Multiskan™ GO instrument (Thermo Fisher Scientific Inc. Waltham, MA, USA).

PCR primers and reagent kits. The reagents used for mRNA isolation (TaqMan Gene Expression Cells-to-Ct™) and PCR (TaqMan Gene Expression Assays) were purchased from Applied Biosystems (Foster City, CA, USA). Assays were conducted according to the manufacturer's instructions (26). For PCR comparison of gene expression, we used Postn (TaqMan Gene Expression Assays; Assay ID: Mm01284919_m1) for periostin and *SEMA3A* (Assay ID: Mm00436469_m1) for semaphorin-3A, with *18S* ribosomal RNA (Mm18s: Assay ID: Mm03928990_g1) used as a housekeeping gene to normalize RNA loading.

mRNA isolation and quantitative PCR. After incubation for 2 h at 37˚C, total RNA was isolated from MC3T3-E1 cells using 50 μl of a lysis solution (P/N4383583). Each sample of total RNA was subjected to reverse transcription (RT) using a 20 × RT enzyme mix and a 2 × RT buffer with a T100 thermal cycler (Bio-Rad Co., Hercules, CA, USA). After reverse transcription, the cDNA templates were amplified with PCR using TaqMan Gene Expression Assays, PCR primers and RT master mix . Predesigned and validated gene-specific TaqMan Gene Expression Assays (26-28) were duplicated for quantitative PCR, based on the manufacturer's protocol. PCR assays were conducted as follows: 10 min denaturation at 95˚C, 40 cycles of 15 s, denaturation at 95˚C, and 1 min annealing and extension at 60˚C. Samples were analysed using an ABI Prism 7900HT Fast RT-PCR System (Applied Biosystems) (28, 29). Relative quantification

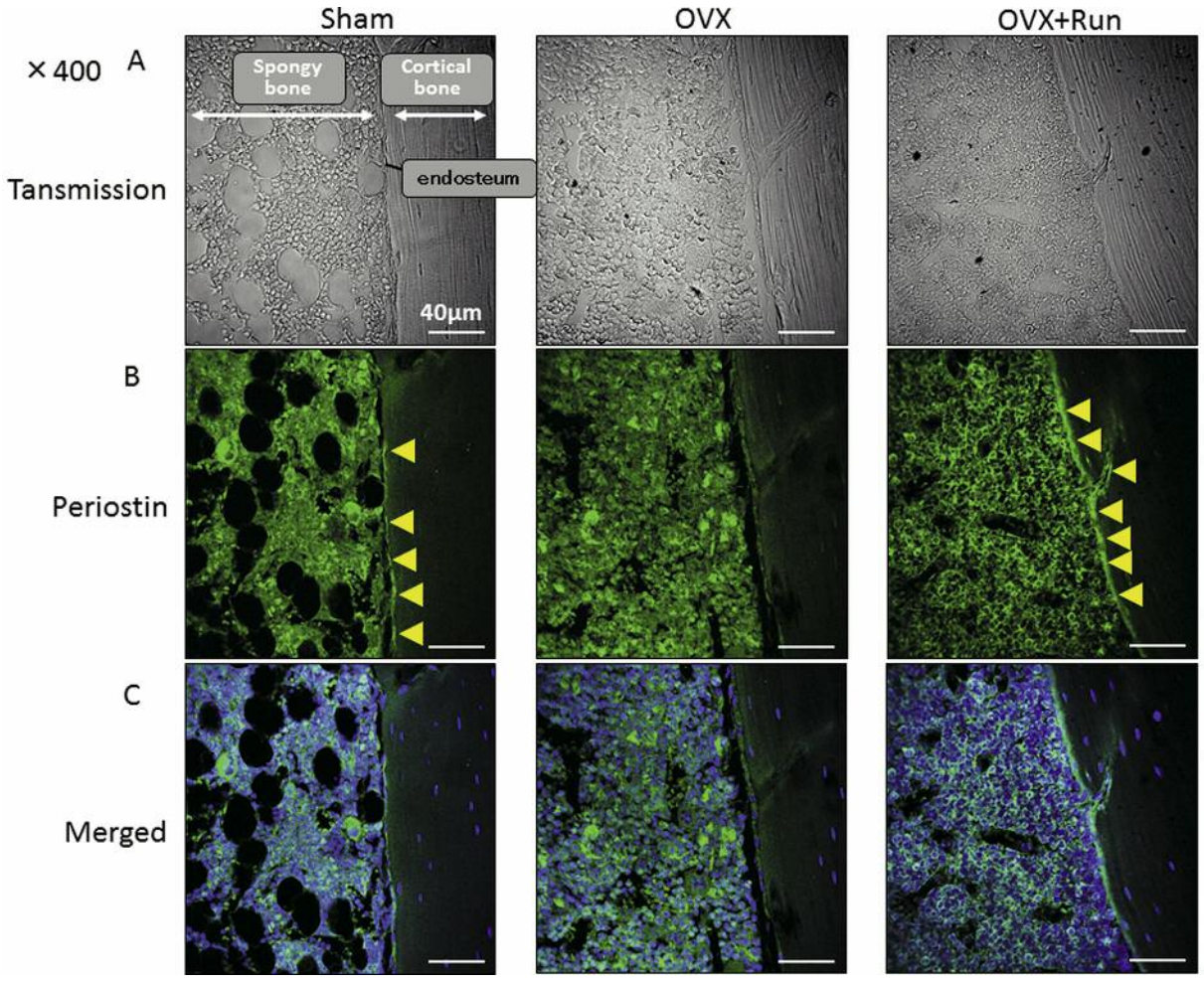

Figure 2. *Immunohistological staining for periostin expression in tibias. Mice were randomly divided into three groups of five mice/group: sham operation, ovariectomy (OVX) and ovariectomy plus treadmill training for 10 weeks (OVX+Run). The animals were sacrificed 24 h after the last exercise session, and tibias were immediately excised for immunohistological analysis. Digital images were obtained using a confocal laser scanning microscope at ×400 magnification. Scale bars=40 μm. A: Transmitted light image from around the tibial endosteum. B: Chromatic image of staining with periostin antibody, green indicates expression of periostin. Arrowheads show where periostin develops in the tibial endosteum. C: Merged image, blue shows nuclei stained by 4',6-diamidino-2-phenylindole.*

studies (30) were prepared from collected data (threshold cycle numbers) with ABI Prism 7900HT Sequence-Detection System (SDS) software v. 2.3 (Applied Biosystem).

Statistical analysis. Data are expressed as means±SD. All assays were repeated three times to ensure reproducibility. Statistical significance of differences between the control and experimental groups was analysed by one-way analysis of variance followed by a Scheffé test. A *p*-value of less than 0.05 was considered statistically significant.

Results

Histochemistry of tibia bone tissue. To identify the effects of exercise load on trabecular bone architecture, proximal tibias were examined with an optical microscope (Figure 1A). The slices were stained with TRAP and osteoclasts were counted (dark purple cell indicated by an arrow in Figure 1A). The blue areas in Figure 1A are epiphyseal cartilage and the pink areas are trabecular bone. The quantity of trabecular bone was lower in the OVX group compared to the sham group, but seemed to remain unchanged in the OVX+Run group. The osteoclasts within the *spongiosa* in the proximal tibia were counted (Figure 1B). Compared to animals of the sham group, the number of TRAP-positive osteoclasts was significantly higher in animals in the OVX group. Compared to OVX animals, there were significantly fewer osteoclasts in OVX+Run mice.

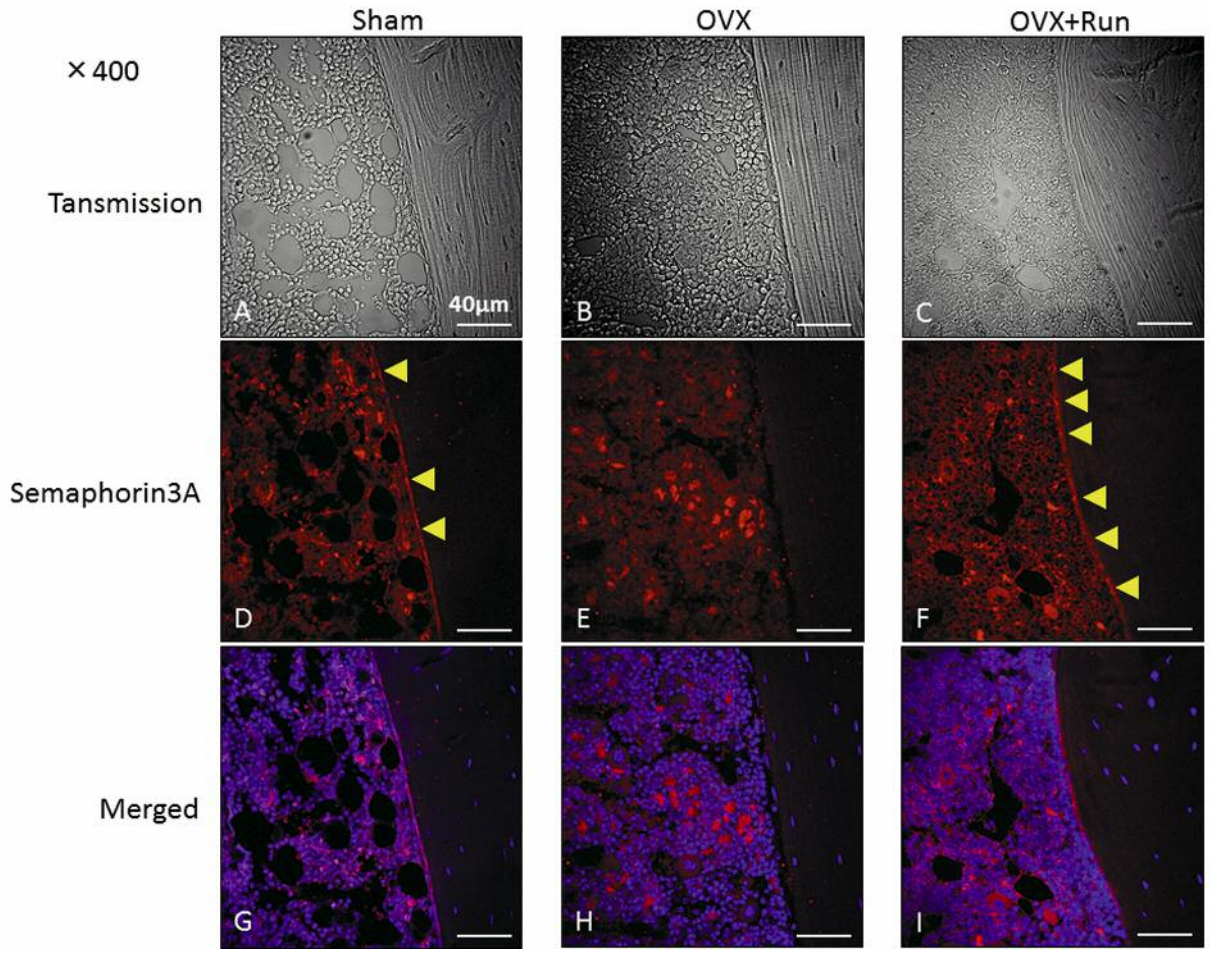

Figure 3. *Immunohistological staining for semaphorin-3A expression in tibias. Digital images were obtained using a confocal laser scanning microscope, photographed at ×400 magnification. Scale bars=40 μm. A: Transmitted light image from around the tibial endosteum. B: Chromatic image of staining with semaphorin-3A antibody, red indicates expression of semaphorin-3A. Arrowheads show where semaphorin-3A develops in the tibial endosteum. C: Merged image, blue shows nuclei stained by 4',6-diamidino-2-phenylindole.*

Periostin and semaphorin-3A expression. We examined whether exercise load affected the expression of periostin and semaphoring-3A expression in the tibias. Figures 2 and 3 show immunohistochemical chromatic images. Periostin (Figure 2) and semaphorin-3A (Figure 3) expression along the endosteum in both the sham and OVX-Run groups was greater than that in the OVX group.

Influence of mechanical stress load on periostin and semaphorin-3A expression. Experiments were conducted to examine the influence of a mechanical stress load on the expression of periostin and semaphorin-3A by MC3T3-E1 cells *in vitro*. The protein content in culture supernatants was examined by ELISA. As shown in Figure 4A, centrifugation, both at 16 ×*g* and at 64 ×*g*, resulted in a significantly higher periostin level in the culture supernatant after 24 h compared with uncentrifuged cells. Centrifugation at 64 ×*g* significantly increased the semaphorin-3A level in the supernatant after 48 h (Figure 4B). Additionally, experiments were conducted to examine whether the mechanical stress load affected mRNA expression in MC3T3-E1 cells. As shown in Figure 4C and D, centrifugation at 16 ×*g* significantly increased periostin and semaphorin-3A mRNA expression in MC3T3-E1 cells.

Influence of periostin on semaphorin-3A. Experiments were conducted to examine the influence of periostin on semaphorin-3A expression in MC3T3-E1 cells *in vitro*. The protein content in culture supernatants were examined by ELISA. As shown in Figure 5A, 1.0 ng/ml of periostin significantly increased the semaphorin-3A level in the culture supernatant of MC3T3-E1 cells after 24 h and 48 h. The lower 0.1 ng/ml periostin dose significantly increased the semaphorin-3A level after 48 h.

Experiments were conducted to examine whether periostin would affect semaphorin-3A mRNA expression in MC3T3-E1

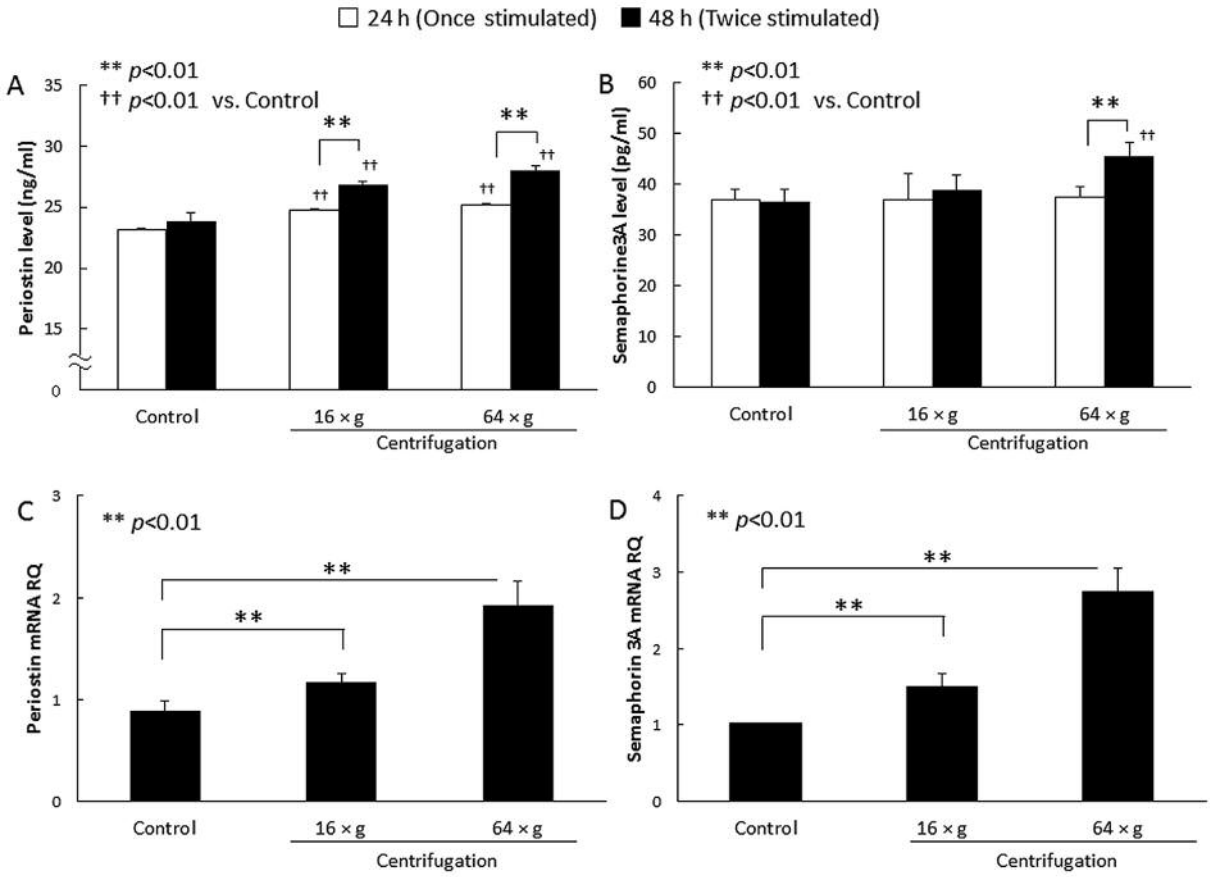

Figure 4. *Mechanical stress effect on MC3T3-E1 cells. MC3T3-E1 cells seeded in 24-well plates in triplicate were burdened by the mechanical stress of centrifugation at 16 ×g or 64 ×g for 15 min, or not (control). The culture supernatants and cells were collected for enzyme-linked immunosorbent assay (ELISA) and real-time reverse transcription polymerase chain reaction (PCR). A: Periostin protein expression by ELISA. B: Semaphorin-3A protein expression by ELISA. C: Periostin mRNA expression by PCR. D: Semaphorin-3A mRNA expression by PCR. Differences statistically significant at **p<0.01; ††p<0.01 vs. Control. Data are expressed as means±standard deviation.*

cells. As shown in Figure 5B and C, periostin and semaphorin-3A mRNA expression in the MC3T3-E1 cells after 24 h significantly increased in a dose-dependent manner.

Discussion

The main objective of our study was to elucidate the influence of mechanical stress on bone matrix protein in a murine model of postmenopausal osteoporosis and in osteoblast-like MC3T3-E1 cells. Firstly, we experimented with OVX mice to verify whether an exercise load can inhibit the process of osteoporosis. Tibia specimens were stained with TRAP and the osteoclasts in the proximal tibia were counted. The number of osteoclasts was significantly lower in the OVX+Run group compared with the OVX group (Figure 1). Bone remodelling consists of two phases, the removal of mineralized bone by osteoclasts, and the formation of new bone by osteoblasts. Therefore, the imbalance of these two cellular functions plays an essential role in skeletal diseases such as osteoporosis (31). Bone-resorbing osteoclasts are formed from a monocyte/macrophage lineage under the strict control of bone-forming osteoblasts (32). Substances known to play major roles in the regulation of osteoclast differentiation include macrophage colony-stimulating factor, receptor activator of nuclear factor-κB ligand and osteoprotegerin produced by osteoblasts. Osteoclast differentiation is tightly regulated by osteoblasts through several mechanisms. It has been reported that mechanical stimulation leads to expression of periostin in bone tissue (33, 34). However, the influence of mechanical stimulation on semaphorin-3A expression, to our knowledge, has not been studied. Therefore, our second experiment was planned to confirm whether a mechanical load affected the expression of the biologically-active substances, periostin and semaphorin-3A.

As shown in Figures 2 and 3, periostin and semaphorin-3A appeared along the tibial endosteum in the OVX+Run group but not in the OVX group. Anatomically, the endosteum is a thin layer of connective tissue that lines the surface of the

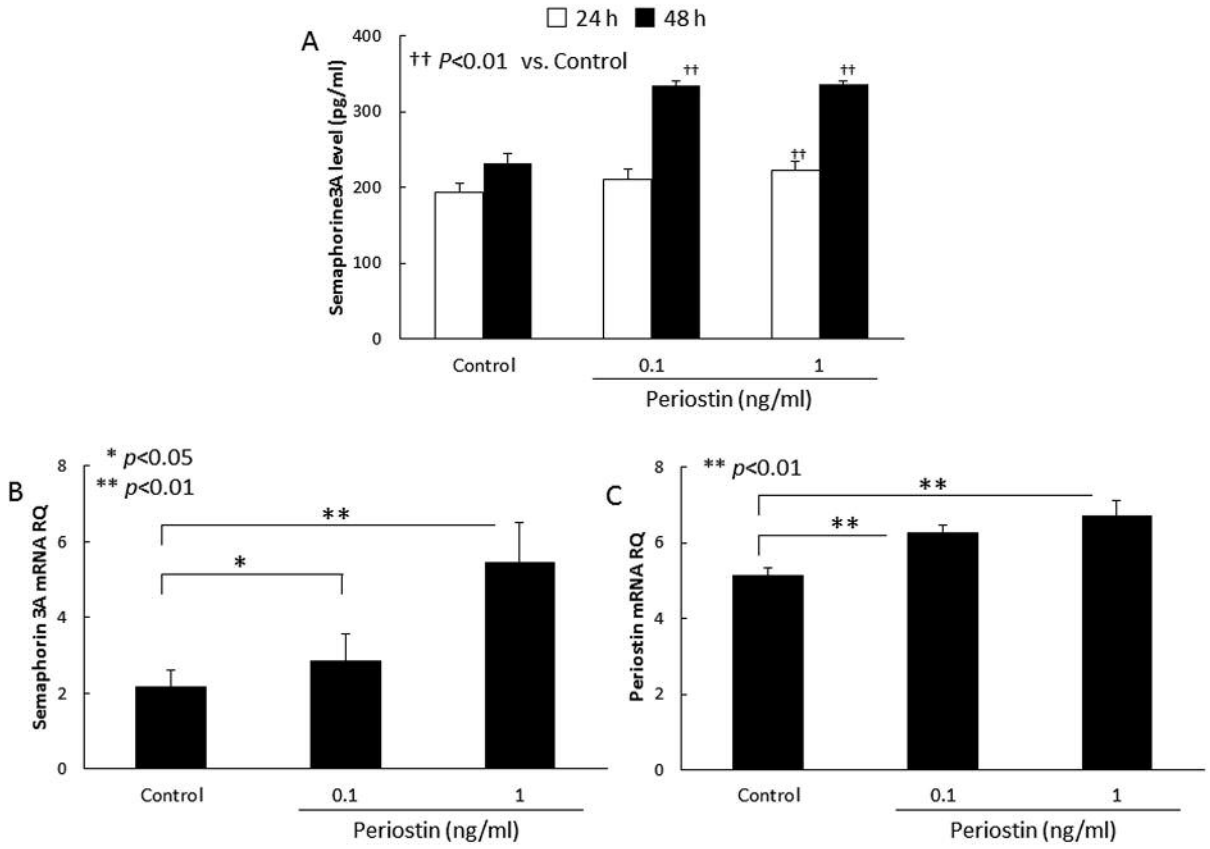

Figure 5. *Effect of treatment with periostin on MC3T3-E1 cells MC3T3-E1 cells were resuspended at a density of 5×10^5 cells/ml in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated foetal calf serum in 24-well plate in triplicate and cultured with 0.1 or 1.0 ng/ml periostin. After 24 or 48 h, culture supernatants were examined with enzyme-linked immunosorbent assay (ELISA) and real-time reverse transcription polymerase chain reaction (PCR). A: Semaphorin-3A protein expression by ELISA. B: Semaphorin-3A mRNA expression by PCR. C: Periostin mRNA expression by PCR. Differences statistically significant at *p<0.05, **p<0.01; ††p<0.01 vs. control. Error bars denote standard deviation.*

bony tissue forming the medullary cavity of long bones. The width of the bone increases as osteoblasts lay new bone tissue at the periosteum. To prevent the bone from becoming unnecessarily thick, osteoclasts resorb the bone from the endosteal side (35). Therefore, localization of periostin and semaphorin-3A along the endosteum may account for the lower number of osteoclasts in the exercised mice.

Periostin is a matrix protein belonging to a fasciclin family that has been identified in a mouse osteoblast cell line. Expression of periostin has been recognized during embryogenesis and in adult connective tissues subjected to mechanical stress (15, 36). Periostin can crosslink with other extracellular matrix proteins, such as collagen I, fibronectin and tenascin-C (37). Horiuchi *et al*. found that periostin-null mice had defective collagen cross-links and lower resistance to mechanical stress (11). Interaction of integrins with matrix proteins such as periostin activates intracellular signaling. These signaling pathways appear to enhance osteoblast differentiation and bone formation *via* Wnt/β-catenin signalling (38). Semaphorin-3A activates also osteoblasts through the Wnt/β-catenin signaling pathway, inhibiting osteoclast differentiation (16). Our results suggest that mechanical load may increase expression of semaphorin-3A in bone tissues.

It is known that the activity of osteocytes and osteoblasts are affected by mechanical stimulation (39, 40). Therefore, we observed the influence of a mechanical load *in vitro* as demonstrated by periostin and semaphorin-3A levels in the osteoblast-like cell line, MC3T3-E1. As shown in Figure 4, the expression of periostin and semaphorin-3A protein and mRNA was significantly enhanced with higher centrifuge speed. The higher mRNA levels suggest that the mechanical load actually increased protein production in osteoblasts rather than simply causing extravasation under pressure of an already preformed substance. Furthermore, the increase in periostin expression was seen earlier than that of semaphorin-3A (Figures 4A and B), indicating that periostin may be upstream in a cascade leading to semaphorin-3A production.

However, the relationship between periostin and semaphorin-3A is still not very clear. Therefore, the final experiment was designed to determine the influence of periostin on semaphorin-3A production by osteoblasts. As shown in Figure 5, the semaphorin-3A level cell culture supernatant and its mRNA expression in MC3T3-E1 cells both increased in a dose-dependent manner when treated with periostin. This further supports the idea that periostin promotes semaphorin-3A production. It is known that periostin activates the nuclear factor-kappa B pathway in an autocrine manner (41), and it may regulate Wnt/β-catenin signalling by autocrine or paracrine mechanisms (41). The fact that periostin mRNA expression also increased with added periostin suggests an autocrine effect on the osteoblasts.

Our results support the concept of a positive correlation between exercise and inhibition of osteoclasts in post-menopausal osteoporosis. Mechanical forces from exercise appear to increase the expression of periostin and semaphorin-3A, which in turn jointly promote activation and differentiation of osteoblasts through Wnt/β-catenin signalling. Actual exercise produces complex mechanical effects such as repetitive compression or shearing forces (42). Therefore, it is unknown whether other types of mechanical stimulation cause a phenomenon similar to what we observed. Additionally, since periostin is produced not only by osteoblasts but also by other cells types (43), further investigation of non-osteoblast cell types is needed. In summary, although it should be noted that the effect of a mechanical load on semaphorin-3A expression has yet to be conclusively proven, we speculate that it increases periostin production in osteoblasts and that periostin then inhibits osteoclast differentiation through its effects on semaphorin-3A expression.

Conflicts of Interest

The Authors declare that there is no conflict of interest regarding this study.

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