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Expression and Dynamics of Podoplanin in Cultured Osteoblasts with Mechanostress and Mineralization Stimulus

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This study investigates the significance of the expression and dynamics of podoplanin in mechanostress and mineralization in cultured murine osteoblasts. Podoplanin increased in osteoblasts subjected to straining in non-mineralization medium, suggesting that the mechanostress alone is a podoplanin induction factor. In osteoblasts subjected to vertical elongation straining in the mineralization medium, the mRNA amounts of podoplanin, osteopontin, and osteocalcin were significantly larger than those in cells not subjected to straining, suggesting that mechanostress is the cause of a synergistic effect in the expression of these proteins. In osteoblasts in the mineralization medium, significant increases in osteocalcin mRNA occurred earlier in cells subjected to straining than in the cells not subjected to straining, suggesting that the mechanostress is a critical factor to enhance the expression of osteocalcin. Western blot and ELISA analysis showed increased podoplanin production in osteoblasts with longer durations of straining. There was significantly less mineralization product in osteoblasts with antibodies for podoplanin, osteopontin, and osteocalcin. There was also less osteopontin and osteocalcin produced in osteoblasts with anti-podoplanin. These findings suggest that mechanostress induces the production of podoplanin in osteoblasts and that podoplanin may play a role in mineralization in cooperation with bone-associated proteins.

Key words: podoplanin, mechanostress, mineralization, anti-podoplanin

I. Introduction

Podoplanin is a mucin-type *O*-glycosylated protein with high sialic acid content. Podoplanin is a platelet aggregation-inducing type I transmembrane protein and occurs in C-type lectin-like receptor-2-mediated platelet activation [15, 16]. Podoplanin has been identified in rat podocytes [5], and is also termed as glycoprotein (gp) 38 of mouse stromal cells of peripheral lymphoid tissue [8], gp36 of human lymphoid tissue [41], RT140 of rat type I alveolar epithelial cells [9], PA2.26 of skin tumor cell lines [29], T1alpha of rat type I alveolar cells [7], and identified as the platelet aggregation-inducing sialoglycoprotein over-expressed on the surface of tumor cells [15]. The absence of podoplanin is lethal to mice because respiratory failure

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occurs at birth due to lung atrophy [28, 32]. In podoplanin knock out mice, the terminal ends of the respiratory tree lack alveolar sacs with podoplanin-positive type I alveolar epithelial cells and there is lung atrophy [32]. The function of podoplanin, particularly in the hard tissue part of somatic tissue, has not been fully established.

In studies of podoplanin of bone-related cells, the earliest description of molecules identical to podoplanin is found in the report of the epitope for gp38 of murine thymic epithelium [8]. Farr *et al*. reported that the predicted gp38 amino acid sequence recognized by a mouse monoclonal antibody from clone 8.1.1 is similar to OTS-8 cloned from the cDNA library, which is an early response protein of murine osteoblast-like MC3T3-E1 cells treated with tumor promoting agent 12-O-tetradecanoylphorbol-13-acetate [8, 22]. Further, podoplanin has been termed E11, which has been established as an antigen recognized by a monoclonal antibody for rat osteoblastic osteosarcoma cell line ROS 17/2.8 cells [36], and the name of E11 has frequently been used for podoplanin in research of bone.

Recently, podoplanin has been established as a marker for osteocytes [11, 30, 33, 39]. Mature osteoblasts and osteocytes in the early differentiation stage express podoplanin at the plasma membrane of cell processes and bone extracts contain large amounts of podoplanin. In primary cultures of osteoblasts, podoplanin-positive cells are absent but appear in increasing with time. Murine osteoblast-like MC3T3-E1 cells and human osteoblast-like MG63 cells commonly express podoplanin and in increasing amounts in mineralization medium [11, 30, 33, 39]. Podoplanin increases in pre-osteocytes on the dendritic processes as the osteoblasts differentiate into osteoid cells or osteocytes, compared with mature markers dentin matrix protein 1 and sclerostin in the osteocyte differentiation marker [2, 21, 39]. Murine long bone osteocyte-like MLO-Y4 and IDG-SW3 cells strongly express podoplanin, more strongly than MC3T3-E1 cells [21, 23, 37]. The differentiation of cultured murine calvarial bone osteoblasts and pre-osteocyte-like MLO-A5 cells into osteocytes in a mineralization medium with β-glycerophosphate and ascorbic acid, and also biomaterial-induced mineralization of osteoblasts into osteocytes are concomitant with an increase in podoplanin [6, 24, 28, 40].

Overall, it has been thought that podoplanin is an early osteocyte marker when osteoblasts differentiate into osteocytes, and that podoplanin may have a role in the metabolism of osteocytes and cell shape formation [3, 11, 18, 19, 23, 24]. However, we have recently reported that there were no morphological anomalies in the development of podoplanin-deficient alveolar bone or teeth in wingless-related MMTV integration site 1 (*Wnt1*)-*Cre*;podoplanin gene $(Pdpn)^{\Delta/\Delta}$ mice: *Wnt1-Cre* transgenic mice, which express Cre recombinase under the control of the *Wnt1* promoter, bred to mice having the homozygous podoplanin-floxed alleles $Pdpn^{fl/fl}$ [32]. In *Wnt1-Cre;*$Pdpn^{\Delta/\Delta}$ mice the teeth and alveolar grow normally, suggesting that podoplanin is not a critical factor in the development of bone. There have been reports that compressive forces induce podoplanin expression in calvarial osteoblasts [26] and that MLO-Y4 cells subjected to fluid flow shear stress show an increase in podoplanin concomitant with elongation of dendrites [39]. However, experimental research focused on the function of podoplanin has not been fully elucidated for the bone metabolism under conditions with elongation stress like orthodontic force to the alveolar bone. It is conceivable that podoplanin is associated with mineralization in bone, depending on the load of the strain by skeletal muscles. The present study aimed to investigate the significance of the expression and dynamics of podoplanin in the mechano-stress and mineralization in cultured murine osteoblasts.

II. Materials and Methods

Cell culture and mechanical strain application

The experimental osteoblasts were induced from the mouse bone marrow osteogenic stromal cells (Cosmo Bio Co., LTD, Tokyo, Japan) with a mouse osteogenesis culture kit (Cosmo Bio) according to the supplier recommendations. Osteoblasts were maintained in Minimum Essential Medium Eagle, Alpha Modification (α-MEM, Sigma-Aldrich Co. LLC., St. Louis, MO) with 10% fetal bovine serum (Biowest, Nuaillé, France). The cells (10,000 cells/well) were seeded on collagen I-coated elastic silicon membranes of 6-well BioFlex Culture Plates (25-mm diameter, 9.6-cm^2 well; Flexcell International Corp, Burlington, NC) in the mineralization medium containing 100 nM dexamethasone, 50 μg/ml, ascorbic acid, and 10 mM β-glycerophosphate for the osteogenic differentiation to test the mineralization. When cells reached about 80% confluence, the cells were subjected to vertical stretching on silicon membrane (5% elongation at 2 cycles/min) using a FX-3000 Flexcell Strain Unit (Flexcell) for five days.

Immunohistochemistry

The silicone membranes with the cells were cut and fixed in 100% ethanol for 30 sec at RT and subsequently immersed in 100% methanol for 30 sec at −20°C, treated with 0.1% goat serum for 30 min at 20°C, and then treated for 8 hr at 4°C with PBS containing 0.1% goat serum and the following primary antibodies (1 μg/ml): hamster monoclonal anti-mouse podoplanin, which recognizes the extracellular domain (AngioBio Co., Del Mar, CA), rabbit anti-mouse osteopontin (Santa Cruz Biotechnology, Inc., Dallas, TX), and rabbit anti-mouse osteocalcin (Proteintech Group, Inc., Chicago, IL). After the treatment with primary antibodies cells were washed three times in PBS for 10 min and immunostained for 0.5 hr at 20°C with 0.1 μg/ml of second antibodies: Alexa Fluor (AF) 488 or 568-conjugated goat anti-hamster IgG (Probes Invitrogen Com., Eugene, OR). The immunostained cells were mounted in 50% polyvinylpyrrolidone solution and examined by fluorescence microscopy (BZ-9000, Keyence Corp., Osaka, Japan), con-

focal laser-scanning microscopy (LSM710, Carl Zeiss, Jena, Germany), or fluorescence microscopy with a Plan Apo lens (Eclipse Ci & DS-Qi2, Nikon, Tokyo, Japan).

Measurement of the area of immunostaining

The podoplanin/osteopontin/osteocalcin-stained areas were measured on five different field-of-view images of immunostained culture using ImageJ (National Institutes of Health, Bethesda, MD). The relative expressed amount of each protein was estimated by the ratio of the immunostained area (%): the podoplanin, osteopontin, and osteocalcin-positive area/the spot scanned are in the culture.

Reverse transcription (RT)-PCR and real-time PCR

The total RNA extraction from the cultured cells was performed with a QIAshredder column and an RNeasy kit (Qiagen, Inc., Tokyo, Japan). Contaminating genomic DNA was removed using DNAfree (Ambion, Huntingdon, UK), and the RT was performed on 30 ng of total RNA at 30°C for 10 min, 55°C for 30 min, and 99°C for 5 min, followed by 30 cycles of PCR for amplification (extension at 72°C for 30 sec, denaturation at 94°C for 30 sec, annealing at 60°C for 90 sec, and followed by final extension at 72°C for 10 min) using the Ex Taq hot start version (Takara Bio Inc., Otsu, Japan) with 50 pM of primer sets for mouse mRNA of β-actin (Mm02619580_g1; Thermo Fisher Scientific, Inc., Yokohama, JAPAN), podoplanin (Mm01348912_g1; Thermo Fisher Scientific), osteopontin (Mm00436767_m1; Thermo Fisher Scientific), and osteocalcin (Mm03413826_mH; Thermo Fisher Scientific), where the specificities had been confirmed by the manufacturer (Thermo Fisher Scientific). The RT-PCR products were separated on 2% agarose gel (NuSieve; FMC, Rockland, ME, USA) and visualized by Syber Green (Takara). The correct size of the amplified PCR products was confirmed by gel electrophoresis and amplification of accurate targets was confirmed by a sequence analysis. To quantify mRNA generation, cDNA samples were analyzed by real-time quantitative PCR. A total of 1 μl of cDNA was amplified in a 25-μl volume of PowerSYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) in a Stratagene Mx3000P real-time PCR system (Agilent Technologies, Inc., Santa Clara, CA, USA), and the fluorescence was monitored at each cycle. Cycle parameters were 95°C for 15 min to activate Taq followed by 35 cycles of 95°C for 15 sec, 58°C for 1 min, and 72°C for 1 min. For the real-time analysis, two standard curves were constructed from amplicons for both the β-actin and target genes in three serial 4-fold dilutions of cDNA. The β-actin or target gene cDNA levels in each sample was quantified against β-actin or the target gene standard curves by allowing the Mx3000P software to accurately determine each cDNA unit. Finally, target gene cDNA units in each sample were normalized to β-actin cDNA units. Then, relative target gene expression units were expressed as arbitrary units, calculated according to the following formula: relative target gene expression units = target gene cDNA units/β-actin cDNA units.

Immunoprecipitation and western-blot analysis

Osteoblasts originating from bone marrow (10,000 cells/well, Cosmo Bio Co., LTD) were cultured in the mouse osteogenesis culture kit (Cosmo Bio) in 6-well plates. When cells reached about 80% confluence, the cells were cultured with stretching as described above, harvested by a cell scraper and solubilized in 1 ml of Pierce IP lysis buffer (Thermo Scientific Inc., Rockford, IL) in a 1.5-ml eppendorf microcentrifuge tube by vortexing for 5 min on ice. The lysate of the whole cell protein was centrifuged for 10 min at 8,000 × g and the supernatant was separated. A 1.0-ml portion of the supernatant of the whole cell lysate with the protein concentration adjusted to 0.1 mg/ml was mixed with a 10-μl volume of Protein G-Agarose beads (Roche Diagnostics Inc., Indianapolis, IN) and hamster anti-mouse podoplanin (0.1 μg/ml; AngioBio). The antibody is an anti-gp38 monoclonal antibody derived from clone 8.1.1 [2, 13]. The binding affinity of hamster IgG to protein-G is weak (Roche Diagnostics), giving rise to the limited influence of remaining IgG on beads on the immuno-blotted membrane. After the immunoprecipitation, the mixture was centrifuged and the beads were separated, and mixed with a 50-μl volume of Sample Buffer (Bio-Rad Laboratories Inc., Hercules, CA). A 15-μl volume of sample was loaded on 15% polyacrylamide gel by electrophoresis with Mini-protean III (Bio-Rad), and the separated proteins were transferred onto a PVDF membrane (Life Technologies Japan Ltd.). After blocking of the PVDF membrane by 5% skimmed milk for 3 hr at 20°C, the membrane was treated with a blocking agent containing 0.1 μg/ml hamster anti-podoplanin (AngioBio) for 2 hr at 20°C, and with peroxidase-conjugated goat anti-hamster (0.1 μg/ml) for 1 hr at 20°C, before being visualized by the substrate of a Vector Elite ABC kit and a Vector VIP kit (Vector Laboratories, Burlingame, CA) or Immun-Star WesternC chemiluminescence Kit (Bio-Rad).

Enzyme-linked immunosorbent assay (ELISA)

Osteoblasts originating from the bone marrow (10,000 cells/well, Cosmo Bio) forming 80% confluent monolayers in the 6-well plates were cultured with stretching as described above and harvested by a cell scraper and solubilized in 100 μl of cell lysis buffer (Cell Signaling Technology, Danvers, MA). The lysate of the whole cell protein (1 mg/ml) was diluted 5-fold with 0.1 M carbonate buffer (pH 9.6) and placed on 96-well microtitration plates for 12 hr at 4°C. After blocking with 5% skimmed milk for 3 hr at room temperature, the plate was treated with 0.1 μg/ml hamster anti-podoplanin (AngioBio) for 3 hr at 20°C and with peroxidase-conjugated goat anti-hamster IgG (0.1 μg/ml) for 1 hr at 20°C, and then visualized by an ABTS peroxidase substrate system (SeraCare Life Sciences, Inc.

[KPL]) in the plates at 37°C and absorbance changes at 405 nm were measured by a microplate reader. Cells treated with only a second antibody served as blanks. The amounts of podoplanin production in the cells were expressed as the mean absorbance of peroxidase metabolizing substrate of six wells.

Antibody-dependent cytotoxicity test

Before investigating the effect of anti-podoplanin in the mineralization, the viability of osteoblasts (Cosmo Bio) cultured with antibodies were tested. Cells were cultured in 96-well microplates in the mouse osteogenesis culture kit (Cosmo Bio) containing 1 and 2 μg/ml rat anti-mouse podoplanin (PMab-1; Medical & Biological Laboratories Co., LTD, Nagoya, JAPAN) [12], rabbit anti-mouse osteopontin (Santa Cruz Biotechnology), rabbit anti-mouse osteocalcin (Proteintech Group), mouse anti-actin (Santa Cruz Biotechnology), and rat/rabbit isotype control IgGs (Santa Cruz Biotechnology) for 10 and 20 days. The cell viability test was performed using water soluble tetrazolium salts WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium of Cell Counting Kit-8 (Dojindo Molecular Technologies, Inc., Tokyo, Japan). The nicotinamide adenine dinucleotide produced by intracellular dehydrogenase in mitochondria reduces WST-8 to orange formazan dye via 1-methoxy-5-methylphenazinium methylsulfate. Since the amount of formazan dye as a reduction product of WST-8 is proportional to the number of cells that are alive, that number was estimated with optical density measurements at the 450 nm wavelength by a microplate reader (SpectraMax Paradigm, Molecular Devices Corp, Tokyo, JAPAN). The number of alive osteoblasts cultured with antibodies were expressed by the absorbance ratio (%): absorbance of the culture with antibodies/absorbance of the culture without antibodies.

Mineralization assay

Osteoblasts originating from bone marrow (10,000 cells/well, Cosmo Bio) were seeded in type 1 collagen-coated 24-well plates (Asahi Glass Co., LTD., Shizuoka, JAPAN) and cultured for 10 and 20 days in the mouse osteogenesis culture kit (Cosmo Bio) containing the antibodies: 1 and 2 μg/ml rat anti-mouse podoplanin (Medical & Biological Laboratories), rabbit anti-mouse osteopontin (Santa Cruz Biotechnology), rabbit anti-mouse osteocalcin (Proteintech Group), mouse anti-actin (Santa Cruz Biotechnology), and rat/rabbit isotype control IgGs (Santa Cruz Biotechnology). The culture was washed in 10 mM phosphate buffered saline (PBS, pH 7.4) and fixed by 10% formalin-PBS. After washing with PBS the culture was stained by alizarin red s using a Calcified Nodule Staining kit (Cosmo Bio) for 30 min. The stained culture was washed 3 times by distilled water to remove the staining dye and 5% formic acid was added to dissolve the calcified nodules. The plates were shaken for 10 min to elute the alizarin red and the mineralization was estimated with optical density measurements of the solution at the 450 nm wavelength by a SpectraMax Paradigm (Molecular Devices Corp).

Effect of anti-podoplanin on the production of osteopontin and osteocalcin

The production of osteopontin and osteocalcin in osteoblasts (Cosmo Bio) treated with anti-podoplanin was tested. The cells (10,000 cells/well) were seeded in α-MEM in 6-well BioFlex Culture Plates. When cells reached about 80% confluence, the medium was changed to mouse osteogenesis culture kit (Cosmo Bio) containing 1 μg/ml rat anti-mouse podoplanin (PMab-1) [13] for 5 days. The expression of podoplanin, osteopontin, and osteocalcin was investigated by immunostaining using hamster anti-mouse podoplanin (AngioBio), rabbit anti-mouse osteopontin (Santa Cruz Biotechnology), and rabbit anti-mouse osteocalcin (Proteintech Group). The immunostaining analyzed by Image J and RT-PCR for podoplanin, osteopontin, and osteocalcin genes were also performed as described above.

Statistics

All experiments were repeated five times, and the data expressed as mean ± SD. Statistically significant differences ($P < 0.01$) were determined by one-way ANOVA and the unpaired two-tailed Student's *t* test with STATVIEW 4.51 software (Abacus concepts, Calabasas, CA, USA).

III. Results

Expression of podoplanin in cultured osteoblasts subjected to mechanical strain

The osteoblasts cultured in α-MEM were immunostained by anti-podoplanin as well as anti-osteopontin and andi-osteocalcin as positive controls, and the staining intensity increased with elongation straining time (days) in the mineralization medium (Fig. 1). In the quantitative analysis of the immunostaining images (Fig. 2A), the relative immunostained area for podoplanin, osteopontin, and osteocalcin increased with the duration (days) of the elongation straining, and the amounts in the culture at 2 and 3 days were significantly larger than in the unstrained culture. The relative amounts for podoplanin and osteocalcin were significantly larger than the culture without straining at 1 day. In the real time-PCR analysis (Fig. 2B), all of the mRNAs for podoplanin, osteopontin, and osteocalcin increased with time of elongation straining and reached a plateau within three days. The mRNA amount of podoplanin in osteoblasts subjected to straining in mineralization medium was significantly larger than in cells subjected to straining in non-mineralization medium, and the amount in cells subjected to straining in non-mineralization medium was significantly larger than in cells not subjected to straining in mineralization medium. The mRNA amount of osteopontin in osteoblasts subjected to straining for 2–5 days in mineralization medium was significantly larger than in cells in

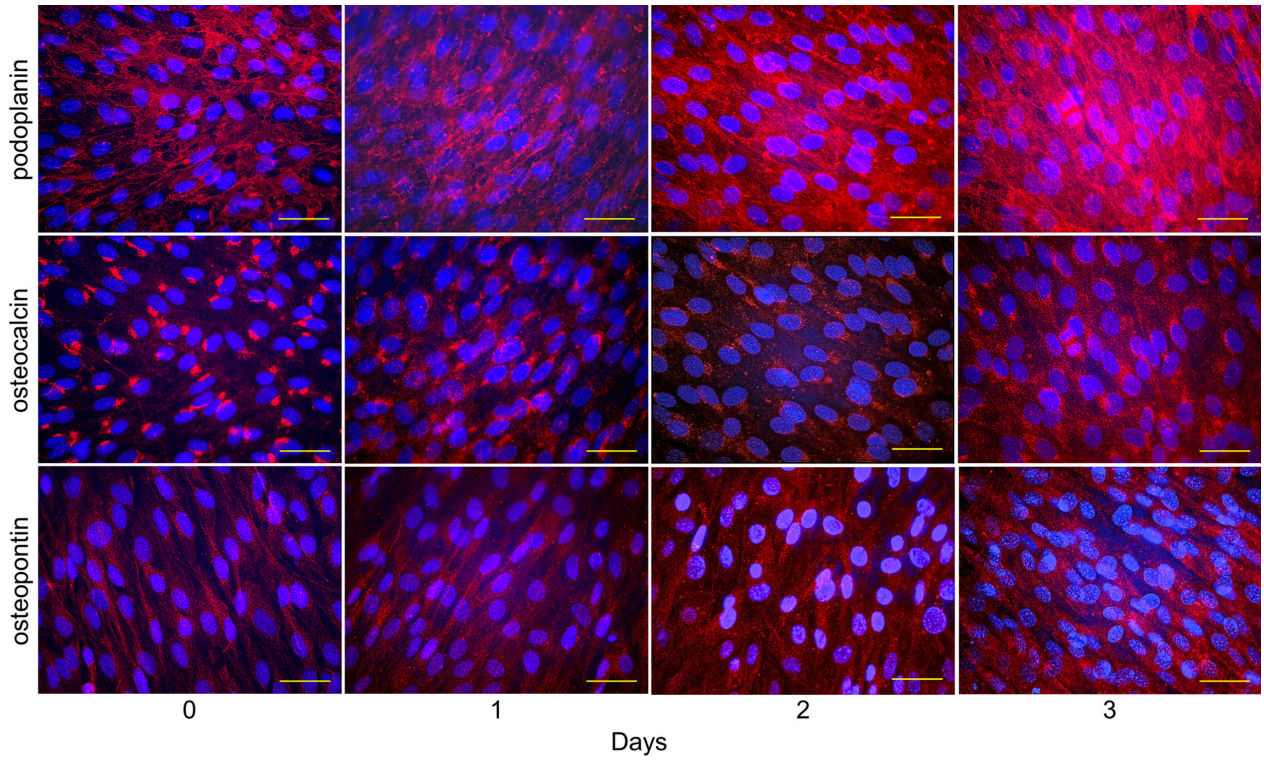

Fig. 1. Immunostaining of cultured osteoblasts subjected to elongation straining. The osteoblasts cultured in the mineralization medium were immunostained (red) by anti-podoplanin, anti-osteopontin, and andi-osteocalcin, and all staining intensities increased with the duration of the elongation straining (days). Nuclei were stained by DAPI. Bars = 100 μm.

mineralization medium not subjected to straining, and the mRNA amount in cells not subjected to straining in non-mineralization medium was similar to the mRNA amount in cells not subjected to straining in mineralization medium. The mRNA amount of osteocalcin in osteoblasts subjected to straining for 3–5 days in mineralization medium was significantly larger than in cells not subjected to straining in mineralization medium, and the amount of mRNA in cells not subjected to straining in mineralization medium was significantly larger than in cells subjected to straining in non-mineralization medium. In the mineralization medium, the significant increase of osteocalcin mRNA occurred earlier in the osteoblasts with straining than in the cells without straining.

In the western blot analysis for the immunoprecipitated protein of cultured osteoblasts subjected to elongation straining (Fig. 3A), one protein band of podoplanin in the range between 37 kDa and 50 kDa increased in visual intensity with duration of straining. There was no cross reaction except for one higher molecular weight band in the range between 75 kDa and 100 kDa. In the chemiluminescence band the density increased with duration of straining (Fig. 3B). In ELISA, the protein production amounts of podoplanin in cultured osteoblasts increased with duration of elongation straining and reached a plateau within three days (Fig. 3C).

Effects of anti-podoplanin on the mineralization in cultured osteoblasts

In the antibody-dependent cytotoxicity test (Fig. 4A), the viability of osteoblasts cultured with rat anti-mouse podoplanin, rabbit anti-mouse osteopontin, rabbit anti-mouse osteocalcin, mouse anti-human actin, and rat isotype control IgG were tested. There were no statistically significantly differences in the numbers of alive osteoblasts cultured in the mineralization medium without any of the antibodies or in any of the cultures with different antibodies at the same concentration and at the same duration of culture as determined by the ANOVA ($P < 0.01$), indicating that the antibodies at the concentration tested here did not affect the cell viability. In the mineralization assay (Fig. 4B, 4C, 4D), mineralization products were significantly smaller in osteoblasts cultured for both 10 and 20 days with antibodies for podoplanin, osteopontin, and osteocalcin than in cells cultured without antibodies or in cultures with mouse anti-actin and rat isotype control IgG at two different concentrations (1 and 2 μg/ml). There were no statistically significant differences between cultures without antibodies or mouse anti-actin and rat isotype control IgG; or between cultures with antibodies for podoplanin, osteopontin, and osteocalcin; as well as between two cultures with the same antibodies at two different concentrations.

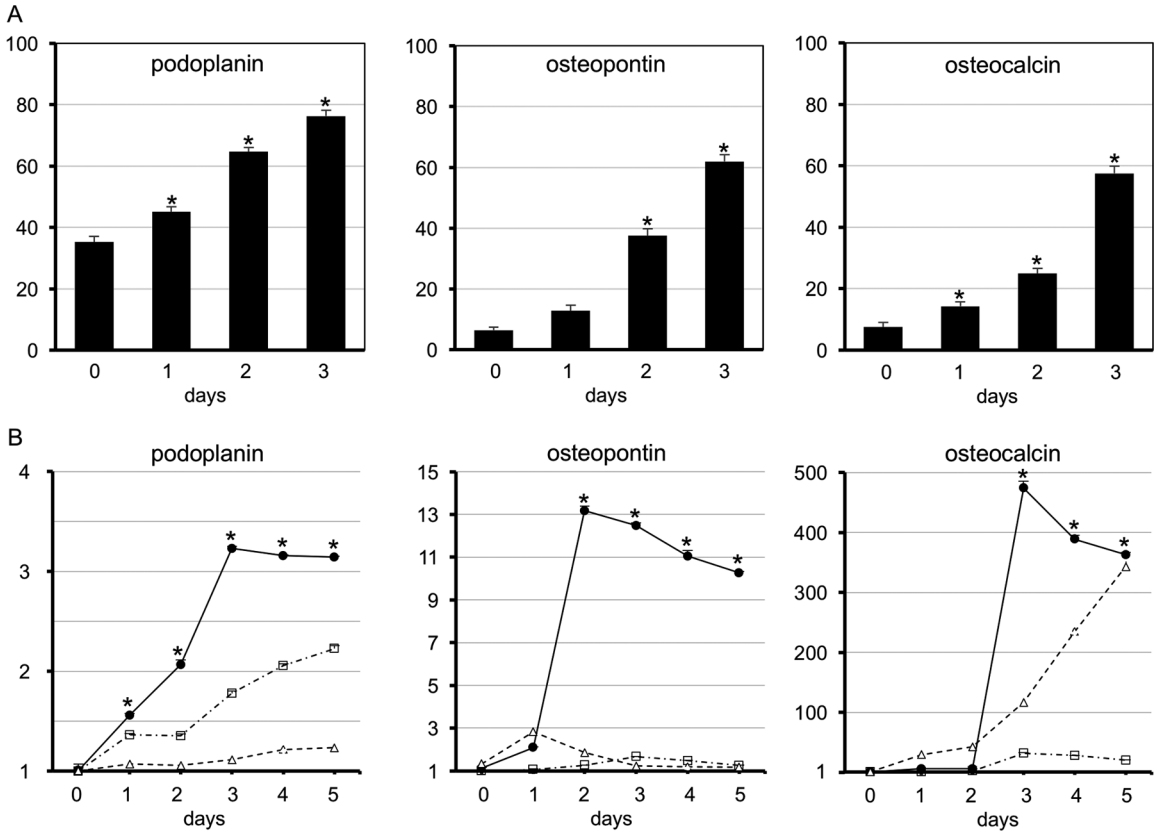

Fig. 2. **A.** Ratio of the immunostained area in cultured osteoblasts subjected to elongation straining. The immunostained area of osteoblasts cultured in the mineralization medium was measured at five different locations in the images using Image J. The relative expressed amounts of each protein were estimated by the ratio of the immunostained area (%): podoplanin, osteopontin, and osteocalcin-positive area/area scanned in the culture. All of the relative expressed amounts of podoplanin, osteopontin, and osteocalcin increased with duration (days) of the elongation straining, and the amounts of culture for 2 and 3 days were statistically significantlly larger than in the unstrained culture. The relative amounts for podoplanin and osteocalcin were significantly larger than the culture at 1 day without straining. *Significantly different in ANOVA ($P < 0.01$). **B.** Real time-PCR analysis for podoplanin, osteopontin, and osteocalcin mRNAs in cultured osteoblasts exposed to elongation straining. The relative amounts of mRNAs were expressed by means of the ratio (%): podoplanin, osteopontin, and osteocalcin cDNA units/β-actin cDNA units. All of the relative amounts of mRNAs increased with duration (days) of elongation straining and reached a plateau within three days. The mRNA of podoplanin of osteoblasts cultured with straining in mineralization medium (closed circles) is significantly larger than in cells cultured with straining in non-mineralization medium (open squares) and without strain in mineralization medium (open triangles). The mRNA of podoplanin of osteoblasts cultured with straining in non-mineralization medium is significantly larger than cells cultured without straining in mineralization medium. The mRNA of osteopontin of osteoblasts cultured with straining for 2–5 days in mineralization medium (closed circles) is significantly larger than in cells cultured with straining in non-mineralization medium (open squares) and without straining in mineralization medium (open triangles). The mRNA of osteopontin of osteoblasts cultured with straining in non-mineralization medium is not significantly different from cells cultured without straining in mineralization medium. The mRNA of osteocalcin of osteoblasts cultured with straining for 3–5 days in mineralization medium (closed circles) is significantly larger than in cells cultured with straining in non-mineralization medium (open squares) and without straining in mineralization medium (open triangles). The mRNA of osteocalcin of osteoblasts cultured without straining in mineralization medium is significantly larger than in cells cultured with straining in non-mineralization medium. In the mineralization medium, the significant increase of osteocalcin mRNA occurred earlier in osteoblasts with straining than in the cells without straining. Values are mean ± SD of data from 6 wells for each group. *Significantly different in ANOVA ($P < 0.01$).

Effects of anti-podoplanin on the production of osteopontin and osteocalcin in cultured osteoblasts

The osteoblasts cultured in α-MEM were immunostained with antibodies specific for podoplanin, osteopontin, and osteocalcin (Fig. 5). The staining intensities for podoplanin and osteopontin seemed to be little increased in osteoblasts by culturing in the mineralization medium while the intensity for osteocalcin increased. In osteoblasts cultured in the mineralization medium, the staining intensities for podoplanin, osteopontin, and osteocalcin are weaker in the cells cultured with anti-podoplanin than in the cells without anti-podoplanin (Fig. 5). The quantitative analysis of the immunostaining images showed that the expressions of podoplanin, osteopontin, and osteocalcin in osteoblasts cultured in the mineralization medium were statistically significantly weaker in the cells with anti-podoplanin than in the cells without anti-podoplanin (Fig. 6A). In osteoblasts without anti-podoplanin, there are no significant differences in the staining intensities for both podoplanin and osteopontin between the cells in α-MEM

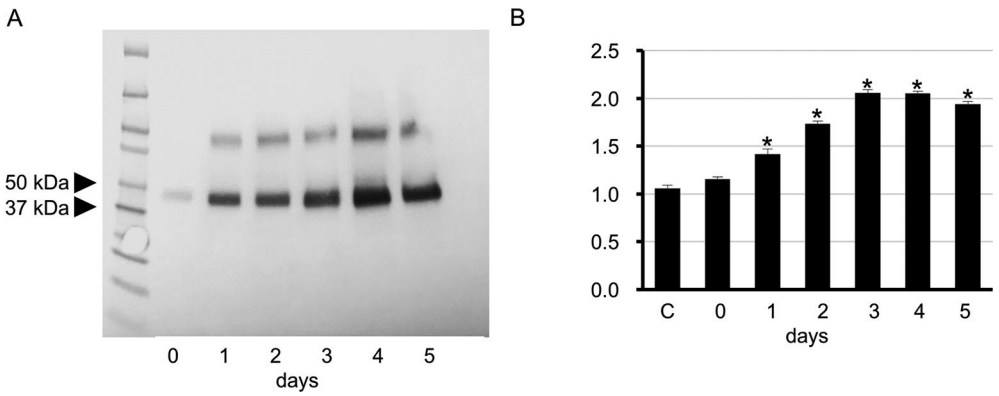

Fig. 3. **A.** Immunoprecipitation and western-blot analysis of the production of podoplanin in cultured osteoblasts with elongation straining. One protein band in the range between the 37 kDa molecular weight (MW) and 50 kDa MW markers is present in each of the lanes and appears to be podoplanin (38 kDa) with sialic acid. The band increased in visual intensity with duration (days) of straining. There is also a hamster IgG band at higher MW between the 75 kDa MW and 100 kDa MW markers. **B.** ELISA for the protein production amounts of podoplanin in cultured osteoblasts with elongation straining. The protein amounts in osteoblasts cultured with straining in mineralization medium increased with the duration of straining (days). C: control amount in osteoblasts cultured in α-MEM. Values are mean ± SD of data from 6 wells for each group. *Significantly different in ANOVA ($P < 0.01$).

and the cells in the mineralization medium, while the staining intensity for osteocalcin is stronger in osteoblasts in the mineralization medium than in the cells in α-MEM. In the real time-PCR analysis, the relative amounts of mRNA of podoplanin, osteopontin, and osteocalcin in osteoblasts cultured with anti-podoplanin were statistically significantly weaker than in the cells without anti-podoplanin. There was little difference in the mRNA amounts of podoplanin and osteopontin between osteoblasts cultured in α-MEM and cells cultured in the mineralization medium, while the mRNA amount of osteocalcin was significantly larger in osteoblasts cultured in the mineralization medium than in cells in α-MEM.

IV. Discussion

It has been reported that osteoblasts with mechanical strain enhance the gene and protein expressions of osteocalcin and osteopontin [26, 35]. It is thought that mechanical strain induces osteogenic effects. In this study, the expression of podoplanin was found in osteoblasts from bone marrow, which was cultured in non-mineralization medium, as well as there was expression of osteopontin and osteocalcin (Figs. 1, 2A); the intensities of the immunostaining with antibodies for podoplanin, osteocalcin, and osteopontin increased with the duration of elongation straining in the mineralization medium, suggesting that the osteoblasts produce podoplanin and increase the produced amounts depending on the elongation strain.

Pre-osteoblasts proliferate actively and express many cell cycle proteins. In the early-stage of maturation, osteoblasts decrease in proliferation and produce type I collagen, TGF-β1, and osteonectin. In the mid-stage of maturation osteoblasts produce alkaline phosphatase and osteopontin. In the late-stage, mature osteoblasts initiate the mineralization of the extracellular matrix. This stage is marked by expression of bone sialoprotein and the latest marker osteocalcin [10, 17, 20, 25, 27]. Recently, type 1 membrane protein podoplanin has been generally recognized as an osteocyte marker. Mature osteoblasts and osteocytes in the early differentiation stage express podoplanin as described above [11, 21, 30, 33, 39]. In the real time-PCR analysis (Fig. 2B), in osteoblasts without straining in the mineralization medium, the late-stage osteoblast marker osteocalcin mRNA increased but the mid-stage marker osteopontin mRNA did not increase. It is likely that the osteoblasts tested here were in the late differentiation stage. The mature osteoblasts/early osteocyte marker podoplanin mRNA increased very little in the osteoblasts without straining in the mineralization medium, suggesting that the osteoblasts tested here were at the stage prior to osteocytes. In the osteoblasts with straining in the non-mineralization medium, podoplanin mRNA increased but osteopontin and osteocalcin mRNAs did not increase, suggesting that the mechanostress alone is the podoplanin induction factor. This may be interpreted to show that the effect of the mechanical strain on the production of bone-matrix proteins should be analyzed in cells with the mineralization stimulus. In osteoblasts strained in the mineralization medium, the mRNA amounts of podoplanin, osteopontin, and osteocalcin were significantly larger than those in cells without straining in the mineralization medium, suggesting that mechanostress gives rise to a synergistic effect on the production of these proteins as a result of the mineralization factor. Further, in osteoblasts in the mineralization medium, a significant increase of osteocalcin mRNA occurred earlier in the cells with straining than in the cells without straining, suggesting that the mechanostress is a critical factor to enhance the expression of osteocalcin in late-stage osteoblasts with the mineralization stimulus.

In the western blot analysis of the immunoprecipitated protein of cultured osteoblasts with exposure to elongation

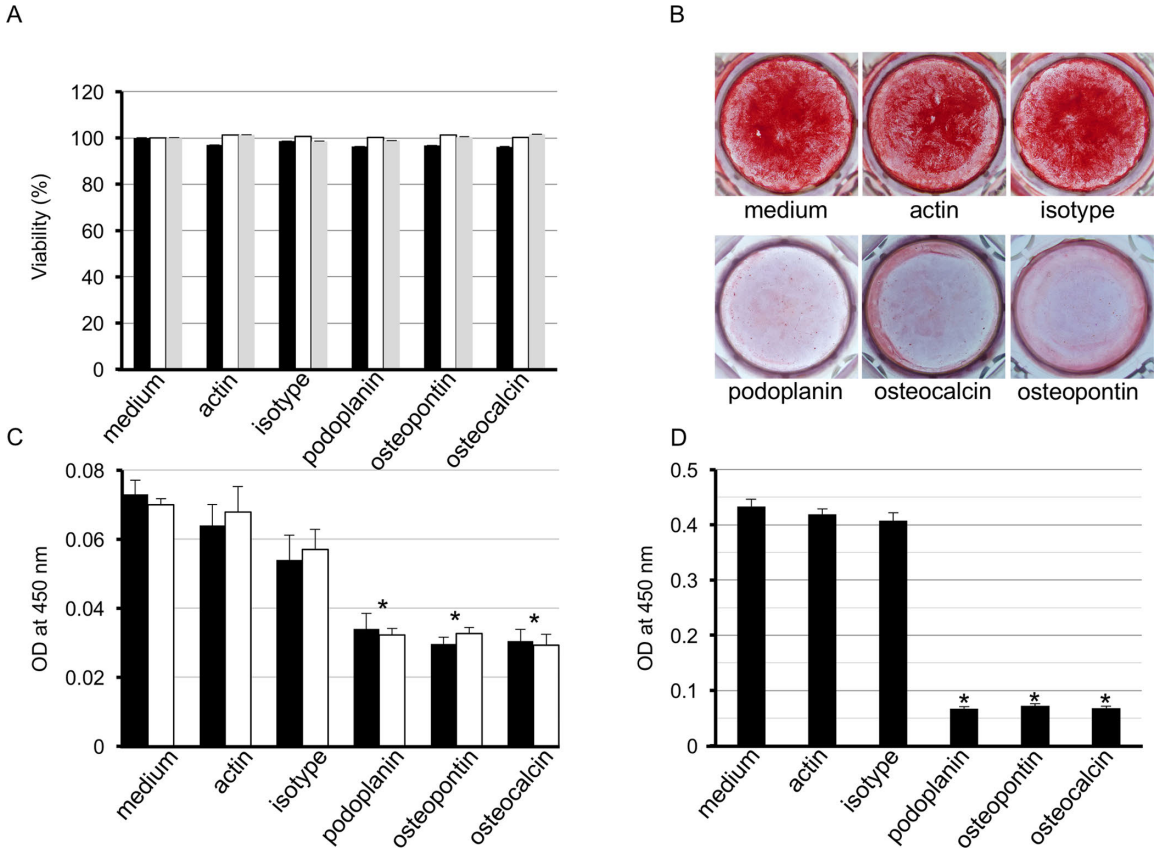

Fig. 4. **A.** Antibody-dependent cytotoxicity test. The viability of osteoblasts cultured with rat anti-mouse podoplanin, rabbit anti-mouse osteopontin, rabbit anti-mouse osteocalcin, mouse anti-actin, and rat isotype control IgG were tested. Cells were cultured in mineralization medium containing 1 μg/ml (open bars) and 2 μg/ml (closed bars) of antibodies for 10 days, and cultured in mineralization medium containing 1 μg/ml of antibodies for 20 days (gray bars). The number of alive cells was estimated by optical density measurements at the 450 nm wavelength for the amount of formazan dye from reduced WST-8. The cell viability with antibodies were expressed by the absorbance ratio (%): absorbance of culture with antibodies/absorbance of culture without antibodies. There were no significant differences among numbers of alive osteoblasts cultured in mineralization medium without antibodies or with different antibodies at the same concentration and at the same culture duration in ANOVA ($P < 0.01$). Values are mean ± SD of data from 6 wells for each group. **B.** Mineralization assay. Alizarin red staining was performed on culture products from osteoblasts cultured in the mineralization medium containing 1 μg/ml of rat anti-mouse podoplanin, rabbit anti-mouse osteopontin, rabbit anti-mouse osteocalcin, mouse anti-actin, and rat isotype control IgG for 20 days. Alizarin red-reacted mineralization products were seen in the culture with no antibodies, and in cultures with mouse anti-actin and rat IgG, but there were no reaction products in cultures with antibodies for podoplanin, osteopontin, and osteocalcin. **C.** Amounts of mineralization products from osteoblasts cultured for 10 days. Alizarin red-reacted mineralization products from osteoblasts cultured with antibodies of 1 μg/ml (closed bars) and 2 μg/ml (open bars) concentrations were dissolved in formic acid and the mineralization was estimated by the optical density measurements at the 450 nm wavelength. Mineralization products were significantly less in cultures with antibodies for podoplanin, osteopontin, and osteocalcin than in culture with no antibodies and in cultures with mouse anti-actin, and rat isotype control IgG. There were no significant differences in cultures with no antibodies and with mouse anti-actin and rat isotype control IgG; in cultures with antibodies for podoplanin, osteopontin, and osteocalcin; in two cultures with the same antibody at two different concentrations. Values are mean ± SD of data from 6 wells for each group. *Significantly different from medium, anti-actin, and isotype controls in ANOVA ($P < 0.01$). **D.** Amounts of mineralization products from osteoblasts cultured for 20 days. Alizarin red-reacted mineralization products from osteoblasts cultured with antibodies of 1 μg/ml concentration were dissolved in formic acid and the mineralization was estimated by optical density measurements at the 450 nm wavelength. Mineralization products were significantly less in cultures with antibodies for podoplanin, osteopontin, and osteocalcin than cultures with no antibodies and cultures with mouse anti-actin, and rat isotype control IgG. There were no significant differences among cultures with no antibodies and with mouse anti-actin, and rat isotype control IgG; in cultures with antibodies for podoplanin, osteopontin, and osteocalcin. Values are mean ± SD of data from 6 wells for each group. *Significantly different from medium, anti-actin, and isotype control in ANOVA ($P < 0.01$).

straining, one protein band of podoplanin in the range between 37 kDa and 50 kDa increased in visual and chemiluminescent density with duration of straining and reached a plateau within 3–5 days (Fig. 3A). There was no cross reaction except in one higher molecular weight band in the range between 75 kDa and 100 kDa. It is thought that remaining IgG on beads has little influence on the blot because of poor binding affinity of hamster IgG to protein-G. Mouse podoplanin has a 38 kDa protein binding much sialic acid [5, 8, 41], and the slight increase in molecular weight appears to be due to sialic acid and the higher band to a reaction of the second antibody to the hamster IgG

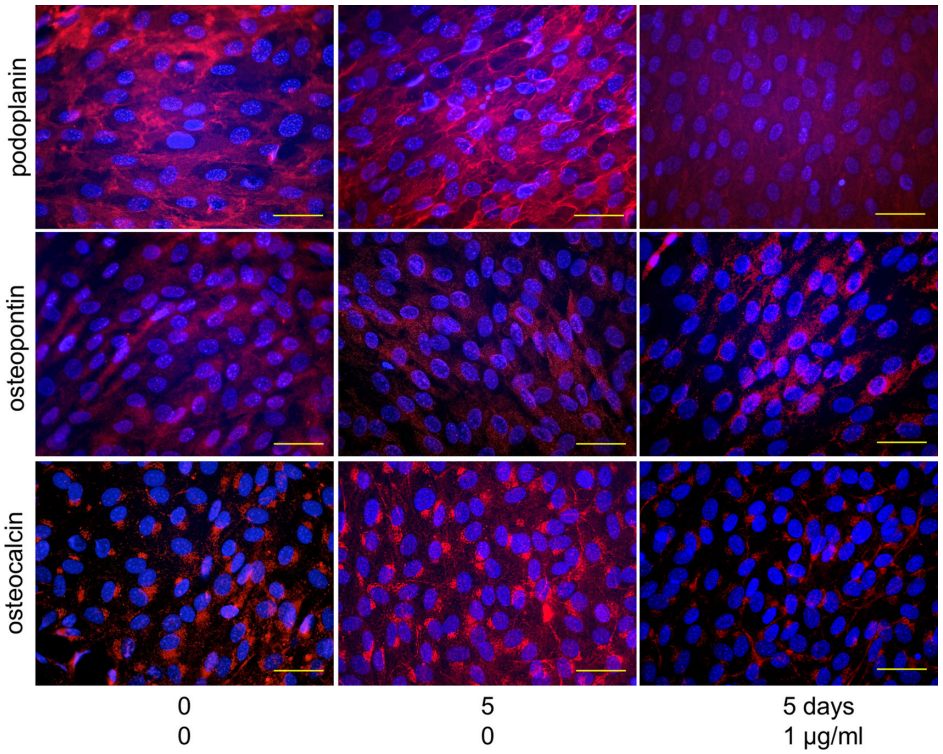

Fig. 5. Immunostaining of osteoblasts cultured with anti-podplanin. The osteoblasts cultured in α-MEM without anti-podoplanin (left lane; 0 day, 0 μg/ml) were immunostained by antibodies specific for podoplanin, osteopontin, and osteocalcin. There are little differences in staining intensities for podoplanin, and osteopontin between osteoblasts cultured in mineralization medium for 5 days (middle lane; 5 days, 0 μg/ml) and cells cultured in α-MEM without anti-podoplanin, while the intensity for osteocalcin increased. In osteoblasts cultured in mineralization medium for 5 days, the staining intensities for podoplanin, osteopontin, and osteocalcin are less in cells cultured with anti-podoplanin (middle lane; 5 days, 1 μg/ml) than in cells without anti-podoplanin (right lane; 5 days, 0 μg/ml). Nuclei were stained by DAPI. Bars = 100 μm.

immunoprecipitated with podoplanin [16]. In ELISA for the lysate of whole cell protein, the produced amount of podoplanin protein in cultured osteoblasts increased with duration of elongation straining and reached a plateau within 3–5 days (Fig. 3B). These results suggest that mechanostress such as elongation straining induces the production of podoplanin protein. In the mature osteoblast MLO-A5 cell line, an increase of podoplanin is concomitant with dendrite formation, mineralization, and RhoA activation [31]. It has been established that podoplanin plays a key role in the elongation and contraction of cell processes by the actin cytoskeleton rearrangement dependent on the binding activity with a cytoplasmic linker protein ezrin through RhoA family signaling [1, 19]. Podoplanin up-regulates a Rho-GTPase activity resulting in ezrin phosphorylation and phosphorylated ezrin mediates a connection of podoplanin to F-actin. The podoplanin expression at the cell membrane promotes the formation of membrane-actin structures critical for the cell shape and also induces plasma membrane extensions based on the cytoskeleton rearrangement [1, 3, 18, 19]. Therefore, podoplanin may function in the cell shape construction of osteocytes such as in dendrite elongation. It is conceivable that podoplanin is associated with mineralization in bone, depending on the load of strain by the skeletal muscle, and that the expression is reinforced by orthodontic mechanostress.

Since the antibodies at the concentration tested here did not affect the cell viability (Fig. 4A), effects of anti-podoplanin on the mineralization, and on the production of osteopontin and osteocalcin were investigated. There was significantly less of mineralization products in both the 10-day and 20-day cultures of osteoblasts cultured with antibodies for podoplanin, osteopontin, and osteocalcin than in cells cultured without antibodies and with antibody controls (Fig. 4B, 4C, 4D). There were no significant differences in three control cultures; in three experimental cultures; or in any of the two cultures with the same antibody at two different concentrations. These results suggest that antibodies for podoplanin inhibited the mineralization in osteoblasts similar to the inhibition by antibodies for osteopontin and osteocalcin, and that podoplanin may be involved in the mineralization in osteoblasts.

Osteoblasts initiate matrix vesicle-mediated mineralization by secreting matrix vesicles consisting of phosphate crystals and non-collagenous proteins such as bone sialoprotein, osteopontin, osteocalcin, and similar, and nuclei of calcium phosphate crystals are formed inside the matrix vesicles [4, 6, 12, 34, 38]. The needle-like, elongated ribbon-shaped crystals assemble radially based on calcium phosphate nuclei and eventually become the globular

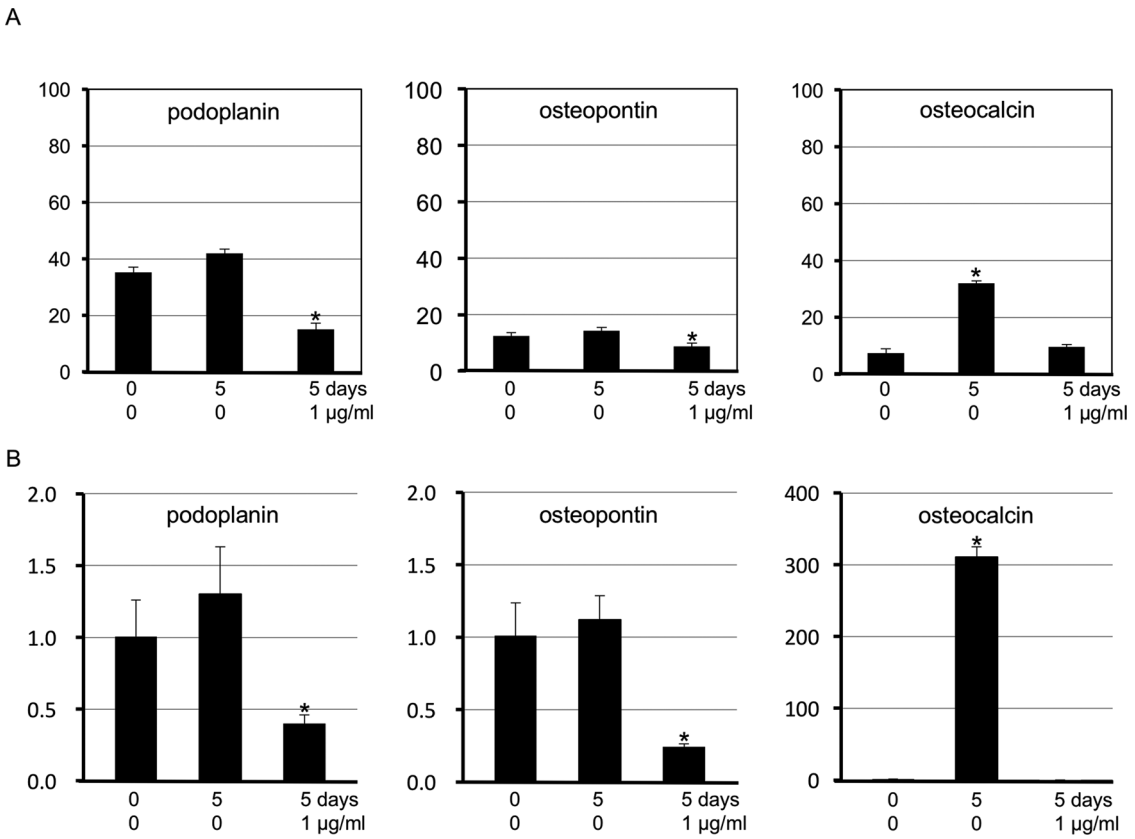

Fig. 6. **A.** Ratio of immunostained areas in osteoblasts cultured with anti-podplanin. The relative amounts of the proteins were estimated by the ratio of the immunostained area. The osteoblasts cultured in α-MEM without anti-podoplanin were immunostained by podoplanin, osteopontin, and osteocalcin (0 day, 0 μg/ml). The staining intensities of podoplanin, osteopontin, and osteocalcin in osteoblasts cultured in mineralization medium for 5 days are statistically significantly weaker in cells with anti-podoplanin (5 days, 1 μg/ml) than in cells without anti-podoplanin (5 days, 0 μg/ml). In osteoblasts without anti-podoplanin, there are no significant differences in staining intensities for both podoplanin and osteopontin between cells in α-MEM (0 days, 0 μg/ml) and cells in mineralization medium (5 days, 0 μg/ml), while the staining intensity for osteocalcin is more in osteoblasts in mineralization medium (5 days, 0 μg/ml) than in cells in α-MEM (0 days, 0 μg/ml). *Significantly different in ANOVA ($P < 0.01$). **B.** Real time-PCR analysis for podoplanin, osteopontin, and osteocalcin mRNAs in osteoblasts cultured with anti-podplanin. The relative amounts of mRNAs were expressed by the means of the ratio (%): podoplanin, osteopontin, and osteocalcin cDNA units/β-actin cDNA units. The mRNA amounts of podoplanin, osteopontin, and osteocalcin in cells cultured with anti-podoplanin (5 days, 1 μg/ml) are statistically significantly smaller than in cells without anti-podoplanin (5 days, 0 μg/ml). In osteoblasts without anti-podoplanin, there are no significant differences in the mRNA amounts for both podoplanin and osteopontin between the cells in α-MEM (0 days, 0 μg/ml) and cells in mineralization medium (5 days, 0 μg/ml), while the mRNA amounts for osteocalcin is more in osteoblasts in mineralization medium (5 days, 0 μg/ml) than in cells in α-MEM (0 days, 0 μg/ml). Values are mean ± SD of data from 6 wells for each group. *Significantly different in ANOVA ($P < 0.01$).

mineralized nodules. The globular crystals are covered by sheaths containing osteocalcin, osteopontin, and bone sialoprotein. When mineralized nodules contact collagen fibers around mineralized nodules, collagen mineralization is propagated from collagen, fiber to fiber [4, 6, 12, 25, 27]. Podoplanin is a sialoprotein containing sialic acid with high binding activity such as for platelet aggregation as described above [14–16]. In this study, the anti-podoplanin inhibited the mRNA and protein production of podoplanin, osteopontin, and osteocalcin in osteoblasts under the condition of mineralization (Figs. 5, 6), suggesting that podoplaninn is associated with the expression of osteopontin and osteocalcin. Osteoblasts showed significant increases in osteocalcin in the mineralization medium but here there was little increase in osteopontin (Figs. 2B, 6). From this, it is thought that the inhibition of podoplanin induced the down-regulation of both osteopontin and osteocalcin in cells in the late-stage of differentiation. Podoplanin may participate in the crystal sheath around mineralized nodules together with osteocalcin and osteopontin. It is conceivable that podoplanin contributes to mineralization in bone, in cooperation with bone-associated proteins.

In conclusion, this study showed that mechanostress induces the production of podoplanin in osteoblasts and that podoplanin in osteoblasts may have a role in the mineralization cooperating with bone-associated proteins.

V. Conflicts of Interest

The authors indicate no potential conflicts of interest.

VI. Acknowledgments

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VII. References

1. Acton, S. E., Farrugia, A. J., Astarita, J. L., Mourão-Sá, D., Jenkins, R. P., Nye, E., Hooper, S., van Blijswijk, J., Rogers, N. C., Snelgrove, K. J., Rosewell, I., Moita, L. F., Stamp, G., Turley, S. J., Sahai, E. and Reis e Sousa, C. (2014) Dendritic cells control fibroblastic reticular network tension and lymph node expansion. *Nature* 514; 498–502.
2. Atkins, G. J., Rowe, P. S., Lim, H. P., Welldon, K. J., Ormsby, R., Wijenayaka, A. R., Zelenchuk, L., Evdokiou, A. and Findlay, D. M. (2011) Sclerostin is a locally acting regulator of late-osteoblast/preosteocyte differentiation and regulates mineralization through a MEPE-ASARM-dependent mechanism. *J. Bone Miner. Res.* 26; 1425–1436.
3. Belkina, N. V., Liu, Y., Hao, J. J., Karasuyama, H. and Shaw, S. (2009) LOK is a major ERM kinase in resting lymphocytes and regulates cytoskeletal rearrangement through ERM phosphorylation. *Proc. Natl. Acad. Sci. U S A* 106; 4707–4712.
4. Bilousova, G., Jun du, H., King, K. B., De Langhe, S., Chick, W. S., Torchia, E. C., Chow, K. S., Klemm, D. J., Roop, D. R. and Majka, S. M. (2011) Osteoblasts derived from induced pluripotent stem cells form calcified structures in scaffolds both in vitroand in vivo. *Stem Cells* 29; 206–216.
5. Breiteneder-Geleff, S., Matsui, K., Soleiman, A., Meraner, P., Poczewski, H., Kalt, R., Schaffner, G. and Kerjaschki, D. (1997) Podoplanin, novel 43-kd membrane protein of glomerular epithelial cells, is down-regu-lated in puromycin nephrosis. *Am. J. Pathol.* 151; 1141–1152.
6. Chen, Z., Wu, C., Yuen, J., Klein, T., Crawford, R. and Xiao, Y. (2014) Influence of osteocytes in the in vitro and in vivo β-tricalcium phosphate-stimulated osteogenesis. *J. Biomed. Mater. Res. A.* 102; 2813–2823.
7. Dobbs, L. G., Williams, M. C. and Gonzalez, R. (1988) Monoclonal antibodies specific to apical surfaces of rat alveolar type I cells bind to surfaces of cultured, but not freshly isolated, type II cells. *Biochim. Biophys. Acta* 970; 146–156.
8. Farr, A. G., Berry, M. L., Kim, A., Nelson, A. J., Welch, M. P. and Aruffo, A. (1992) Characterization and cloning of a novel glycoprotein expressed by stromal cells in T-dependent areas of peripheral lymphoid tissues. *J. Exp. Med.* 176; 1477–1482.
9. Gonzalez, R. F. and Dobbs, L. G. (1998) Purification and analysis of RT140, a type I alveolar epithelial cell apical membrane protein. *Biochim. Biophys. Acta* 1429; 208–216.
10. Granéli, C., Thorfve, A., Ruetschi, U., Brisby, H., Thomsen, P., Lindahl, A. and Karlsson, C. (2014) Novel markers of osteogenic and adipogenic differentiation of human bone marrow stromal cells identified using a quantitative proteomics approach. *Stem Cell Res.* 12; 153–165.
11. Hadjiargyrou, M., Rightmire, E. P., Ando, T. and Lombardo, F. T. (2001) The E11 osteoblastic lineage marker is differentially expressed during fracture healing. *Bone* 29; 149–154.
12. Hasegawa, T., Yamamoto, T., Tsuchiya, E., Hongo, H., Tsuboi, K., Kudo, A., Abe, M., Yoshida, T., Nagai, T., Khadiza, N., Yokoyama, A., Oda, K., Ozawa, H., de Freitas, P. H. L., Li, M. and Amizuka, N. (2017) Ultrastructural and biochemical aspects of matrix vesicle-mediated mineralization. *Jpn. Dent. Sci. Rev.* 53; 34–45.
13. Kaji, C., Tsujimoto, Y., Kato Kaneko, M., Kato, Y. and Sawa, Y. (2012) Immunohistochemical examination of novel rat monoclonal antibodies against mouse and human podoplanin. *Acta Histochem. Cytochem.* 45; 227–237.
14. Kaneko, M. K., Nakamura, T., Kunita, A., Fukayama, M., Abe, S., Nishioka, Y., Yamada, S., Yanaka, M., Saidoh, N., Yoshida, K., Fujii, Y., Ogasawara, S. and Kato, Y. (2017) ChLpMab-23: cancer-specific human-mouse chimeric anti-PDPN antibody exhibits antitumor activity via antibody-dependent cellular cytotoxicity. *Monoclon. Antib. Immunodiagn. Immunother.* 36; 104–112.
15. Kato, Y., Fujita, N., Kunita, A., Sato, S., Kaneko, M. and Osawa, M. (2003) Molecular identification of Aggrus/T1α as a platelet aggregation-inducing factor expressed in colorectal tumors. *J. Biol. Chem.* 278; 51599–51605.
16. Kato, Y. and Kaneko, M. K. (2014) A cancer-specific monoclonal antibody recognizes the aberrantly glycosylated podoplanin. *Sci. Rep.* 4; 5924.
17. Kusuyama, J., Bandow, K., Ohnishi, T., Hisadome, M., Shima, K., Semba, I. and Matsuguchi, T. (2017) Osteopontin inhibits osteoblast responsiveness through the down-regulation of focal adhesion kinase mediated by the induction of low-molecular weight protein tyrosine phosphatase. *Mol. Biol. Cell* 28; 1326–1336.
18. Lan, M., Kojima, T., Murata, M., Osanai, M., Takano, K., Chiba, H. and Sawada, N. (2006) Phosphorylation of ezrin enhances microvillus length via a p38 MAP-kinase pathway in an immortalized mouse hepatic cell line. *Exp. Cell Res.* 312; 111–120.
19. Martín-Villar, E., Megías, D., Castel, S., Yurrita, M. M., Vilaró, S. and Quintanilla, M. (2006) Podoplanin binds ERM proteins to activate RhoA and promote epithelial-mesenchymal transition. *J. Cell Sci.* 119; 4541–4553.
20. Matsuguchi, T., Chiba, N., Bandow, K., Kakimoto, K., Masuda, A. and Ohnishi, T. (2009) JNK activity is essential for Atf4 expression and late-stage osteoblast differentiation. *J. Bone Miner. Res.* 24; 398–410.
21. Meury, T., Akhouayri, O., Jafarov, T., Mandic, V. and St-Arnaud, R. (2010) Nuclear alpha NAC influences bone matrix mineralization and osteoblast maturation in vivo. *Mol. Cell. Biol.* 30; 43–53.
22. Nose, K., Saito, H. and Kuroki, T. (1990) Isolation of a gene sequence induced later by tumor-promoting 12-O-tetradecanoylphorbol-13-acetate in mouse osteoblastic cells (MC3T3-E1) and expressed constitutively in ras-transformed cells. *Cell Growth Differ.* 1; 511–518.
23. Ohizumi, I., Harada, N., Taniguchi, K., Tsutsumi, Y., Nakagawa, S., Kaiho, S. and Mayumi, T. (2000) Association of CD44 with OTS-8 in tumor vascular endothelial cells. *Biochim. Biophys. Acta* 1497; 197–203.
24. Prideaux, M., Loveridge, N., Pitsillides, A. A. and Farquharson, C. (2012) Extracellular matrix mineralization promotes E11/gp38 glycoprotein expression and drives osteocytic differentiation. *PLoS One* 7; e36786.
25. Raouf, A. and Seth, A. (2000) Ets transcription factors and targets in osteogenesis. *Oncogene* 19; 6455–6463.
26. Rath, B., Nam, J., Knobloch, T. J., Lannutti, J. J. and Agarwal, S. (2008) Compressive forces induce osteogenic gene expression in calvarial osteoblasts. *J. Biomech.* 41; 1095–1103.
27. Rogers, M. B., Shah, T. A. and Shaikh, N. N. (2015) Turning bone morphogenetic protein 2 (BMP2) on and off in mesenchymal cells. *J. Cell Biochem.* 116; 2127–2138.
28. Schacht, V., Ramirez, M. I., Hong, Y. K., Hirakawa, S., Feng,

D., Harvey, N., Williams, M., Dvorak, A. M., Dvorak, H. F., Oliver, G. and Detmar, M. (2003) T1a/podoplanin deficiency disrupts normal lymphatic vasculature formation and causes lymphedema. *EMBO J.* 22; 3546–3556.
29. Scholl, F. G., Gamallo, C., Vilar, S. and Quintanilla, M. (1999) Identification of PA2.26 antigen as a novel cell-surface mucin-type glycoprotein that induces plasma membrane extensions and increased motility in keratinocytes. *J. Cell Sci.* 112; 4601–4613.
30. Schulze, E., Witt, M., Kasper, M., Löwik, C. W. and Funk, R. H. (1999) Immunohistochemical investigations on the differentiation marker protein E11 in rat calvaria, calvaria cell culture and the osteoblastic cell line ROS 17/2.8. *Histochem. Cell Biol.* 111; 61–69.
31. Staines, K. A., Prideaux, M., Allen, S., Buttle, D. J., Pitsillides, A. A. and Farquharson, C. (2016) E11/Podoplanin protein stabilization through inhibition of the proteasome promotes osteocyte differentiation in murine in vitro models. *J. Cell. Physiol.* 231; 1392–1404.
32. Takara, K., Maruo, N., Oka, K., Kaji, C., Hatakeyama, Y., Sawa, N., Kato, Y., Yamashita, J., Kojima, H. and Sawa, Y. (2017) Morphological study of tooth development in podoplanin-deficient mice. *PLoS One* 12; e0171912.
33. Vazquez, M., Evans, B. A., Riccardi, D., Evans, S. L., Ralphs, J. R., Dillingham, C. M. and Mason, D. J. (2014) A new method to investigate how mechanical loading of osteocytes controls osteoblasts. *Front. Endocrinol.* 5; 208.
34. Veis, A. (1993) Mineral-matrix interactions in bone and dentin. *J. Bone Miner. Res.* Suppl 2; S493–497.
35. Walker, L. M., Publicover, S. J., Preston, M. R., Said Ahmed, M. A. and El Haj, A. J. (2000) Calcium-channel activation and matrix protein upregulation in bone cells in response to mechanical strain. *J. Cell Biochem.* 79; 648–661.
36. Wetterwald, A., Hoffstetter, W., Cecchini, M. G., Lanske, B., Wagner, C., Fleisch, H. and Atkinson, M. (1996) Characterization and cloning of the E11 antigen, a marker expressed by rat osteoblasts and osteocytes. *Bone* 18; 125–132.
37. Woo, S. M., Rosser, J., Dusevich, V., Kalajzic, I. and Bonewald, L. F. (2011) Cell line IDG-SW3 replicates osteoblast-to-late-osteocyte differentiation in vitro and accelerates bone formation in vivo. *J. Bone Miner. Res.* 26; 2634–2646.
38. Wuthier, R. E. (1989) Mechanism of de novo mineral formation by matrix vesicles. *Connect. Tissue Res.* 22; 27–33.
39. Zhang, K., Barragan-Adjemian, C., Ye, L., Kotha, S., Dallas, M., Lu, Y., Zhao, S., Harris, M., Harris, S. E., Feng, J. Q. and Bonewald, L. F. (2006) E11/gp38 selective expression in osteocytes: regulation by mechanical strain and role in dendrite elongation. *Mol. Cell. Biol.* 26; 4539–4552.
40. Zhu, D., Mackenzie, N. C., Millán, J. L., Farquharson, C. and MacRae, V. E. (2011) The appearance and modulation of osteocyte marker expression during calcification of vascular smooth muscle cells. *PLoS One* 6; e19595.
41. Zimmer, G., Oeffner, F., Von Messling, V., Tschernig, T., Gröness, H. J., Klenk, H. D. and Herrler, G. (1999) Cloning and characterization of gp36, a human mucin-type glycoprotein preferentially expressed in vascular endothelium. *Biochem. J.* 341; 277–284.