博 士 論 文

Expression dynamics of podoplanin in the mineralization of cultured osteoblasts with mechanostress
(機械的ストレスを負荷した培養骨芽細胞の石灰化におけるポドプラニンの発現動態)

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北海道大学
大学院歯学研究科口腔医学専攻

竹 縄　智 紘

Abstract

Here, we report the expression dynamics of podoplanin in cultured bone marrow-derived osteoblasts with mechanical stress and the effect of anti-podolanin on the mineralization. The expression of podoplanin in cultured osteoblasts subjected to vertical elongation straining was significantly higher than that in cells without straining, and the expression increased with duration of elongation time as well as the expression for both osteopontin and osteocalcin tested as positive controls. The podoplanin mRNA amount in cultured osteoblasts subjected to straining in non-mineralization medium was significantly larger than in unstrained cells in mineralization medium. In the western blot analysis for the immunoprecipitated protein and in ELISA for the cell lysate, cultured osteoblasts increased podoplanin production with longer durations of elongation straining and reached a plateau within 3-5 days. There are significantly less of mineralization products in osteoblasts cultured with antibodies for podoplanin, as well as in cells with both antibodies for osteopontin, and osteocalcin as positive controls than in cells with medium only and with phenotype antibody controls. These findings may suggest that mechanostress induces the production of podoplanin, and that podoplanin may contribute to the mineralization in osteoblasts in bone under the circumstances with mechanostress.

Keywords: podoplanin, mechanostress, mineralization, anti-podoplanin

I. Introduction

Podoplanin is a mucin-type *O*-glycosylated protein with high sialic acid content. Podoplanin has been identified in rat podocytes[1)] and also termed a glycoprotein (gp) 38 of mouse stromal cells of peripheral lymphoid tissue[2)], gp36 of human lymphoid tissue[3)], RT140 of rat type I alveolar epithelial cells[4)], PA2.26 of skin tumor cell lines[5)], T1alpha of rat type I alveolar cells[6)], and the platelet aggregation-inducing sialoglycoprotein (Aggrus) overexpressed on the surface of tumor cells[7)]. The function of podoplanin is still not fully elucidated since podoplanin absence is lethal to mice because of the occurrence of respiratory failure at birth[8,9)]. In the studies of bone-related cells, there is the earliest description for molecules identical to podoplanin in the report of the epitope for gp38 of murine thymic epithelium[2)]. 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 a cDNA library of the early response protein derived from murine osteoblast-like MC3T3-E1 cells treated with tumor promoting agent 12-O-tetradecanoylphorbol-13-acetate[10)]. Podoplanin is also termed E11 antigen established as a monoclonal antibody for cell membrane protein of rat osteoblastic osteosarcoma cell line ROS 17/2.8 cells[11)]. Osteocytes are terminally differentiated from

osteoblasts which reside in a mineralized extracellular matrix. 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[12,13,14]. In the primary culture of bone-related cells, podoplanin-positive osteoblasts are absent but increase with time, and osteoblast-like cell lines increase podoplanin amounts with differentiation and mineralization. In the osteocyte differentiation marker genes, podoplanin increases in pre-osteocytes on the dendritic processes as the osteoblast differentiates into an osteocyte, compared with mature markers dentin matrix protein 1 and sclerostin[15,16,17]. In cell lines, the expression amounts of podoplanin is larger in murine long bone osteocyte-like MLO-Y4 cells than in murine osteoblast-like MC3T3-E1 cells and primary osteoblasts, and MLO-Y4 cells subjected to fluid flow shear stress show an increase of podoplanin concomitant with dendricity and elongation of dendrites[17]. The murine osteocyte-like IDG-SW3 cells from long-bone express podoplanin in culture[13], and the differentiation of cultured murine calvarial osteoblasts into osteocytes in mineralization medium with β-glycerophosphate and ascorbic acid is concomitant with an increase of an early osteocyte marker podoplanin[18]. Murine pre-osteocyte-like MLO-A5 cells with ascorbic acid and phosphate promote progressive extracellular matrix mineralization concomitant with podoplanin expression[19]. In the

biomaterials-induced mineralization, osteocytes upregulate podoplanin[8]. There has been also reported that human osteoblast-like MG63 cells express podoplanin like MLO-Y4 and MC3T3-E1, whereas collagen type 1 α1 expression is stronger in osteoblasts than in osteocytes[20]. Overall, it has been thought that podoplanin is an early osteocyte marker when osteoblasts differentiate into osteocytes. Here, we report the expression dynamics of podoplanin under mechanical stress and a role of podoplanin in the mineralization using cultured osteoblasts.

II. Materials and methods

Cell culture and mechanical strain application

Osteoblasts originating from bone marrow (Cosmo Bio Co., LTD, Tokyo, JAPAN) were cultured in a mouse osteogenesis culture kit (Cosmo Bio): maintained in Minimum Essential Medium Eagle, Alpha Modification (α-MEM, Sigma-Aldrich Co. LLC., St. Louis, MO, USA) with 10% bovine serum; and cultured in the mineralization medium containing 100 nM dexamethasone, 50 μg/ml, ascorbic acid, and 10mM β-glycerophosphate for the osteogenic differentiation to test the mineralization. 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, USA). When cells reached about 80% confluence, cells were cultured with vertical stretching (5% elongation at 2 cycles/min) using a FX-3000 Flexcell Strain Unit (Flexcell) for five days.

Immunohistochemistry

Cultured cells were fixed in 100% ethanol for 30 sec at room temperature 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 hrs at 4˚C with PBS containing 0.1% goat serum and the following primary antibodies (1 μg/ml): hamster monoclonal anti-mouse podoplanin (AngioBio Co., Del Mar, CA, USA), rabbit anti-mouse osteopontin (Santa Cruz Biotechnology, Inc., Dallas, TX, USA), and rabbit anti-mouse osteocalcin (Proteintech Group, Inc., Chicago, IL, USA). After the treatment with primary antibodies the sections/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, USA). The immunostained sections were mounted in 50% polyvinylpyrrolidone solution and examined by fluorescence microscopy (BZ-9000, Keyence Corp., Osaka,

JAPAN), confocal 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, USA). 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, followed by 30 cycles of PCR for amplification 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 Bio). 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 1μl of cDNA was amplified in a 25μl 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 15s, 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, Tokyo, JAPAN) 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, USA) 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 x 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, USA) and hamster anti-mouse podoplanin (0.1 μg/ml; AngioBio). 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, USA). 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 IgG (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, USA) 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, USA). The lysate of the whole cell protein (1 mg/ml) was diluted 5-fold with 0.1 M carbonate buffer (pH 9.6) and was placed onto 96-well microtitration plates for 12 h 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 (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 which were estimated with optical density measurements of the solution at the 450nm wavelength by a SpectraMax Paradigm (Molecular Devices Corp).

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 with mineralization medium containing 1 and 2 ug/ml rat anti-mouse podoplanin (PMab-1; Medical & Biological Laboratories Co., LTD, Nagoya, JAPAN) [21], 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 (N = 6). The medium was changed every 2 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 450nm 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 ug/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 10mM 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 450nm wavelength by a SpectraMax Paradigm (Molecular Devices Corp).

Statistics

All experiments 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). The relative immunostained area for podoplanin (Fig. 2A), osteopontin, and osteocalcin increased with 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 significantly increased with time of elongation straining and reached a plateau within three days. The mRNA amount of podoplanin in osteoblasts cultured and subjected to straining in mineralization medium was larger than in cells not subjected to straining in mineralization medium, and the amount in cells subjected to straining in non-mineralization medium was larger than in cells not subjected to straining in mineralization medium. The mRNA amount of osteopontin in

osteoblasts cultured with straining for 2-5 days in mineralization medium was larger than in cells in 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 cultured and subjected to straining for 3-5 days in mineralization medium was 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 larger than in cells subjected to straining in non-mineralization medium.

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 75kDa 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 of 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 significantly differences in the numbers of alive osteoblasts cultured in the mineralization medium without 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 ($P<0.01$). 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.

IV. Discussion

It has been reported that osteoblasts with mechanical strain enhance the gene and protein expressions of osteocalcin and osteopontin[22,23]. 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 the expression of osteopontin and osteocalcin (Fig. 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-like cells used in this study produce podoplanin and increase the production amounts depending on the elongation strain. In the real time-PCR analysis (Fig. 2B), the mRNA amount of podoplanin, and mRNA amounts of osteopontin and osteocalcin tested as positive controls were larger in osteoblasts cultured with straining in mineralization medium than those in cells not exposed to straining in mineralization medium, and the podoplanin mRNA amount was larger in osteoblasts with exposure to straining in non-mineralization medium than in cells not exposed to straining in mineralization medium. These results suggest that mechanostress is stronger factors in the expression of podoplanin in osteoblasts than growth factors for osteogenic differentiation. There were no differences between the osteopontin mRNA amounts in osteoblasts with exposure to straining in non-mineralization medium and in osteoblasts

not exposed to straining in mineralization medium, suggesting that a combination of mechanostress and growth factors is a strong inducer for the production of osteopontin. The osteocalcin mRNA amount in osteoblasts not exposed to straining in mineralization medium is larger than cells with exposure to elongation straining in non-mineralization medium, suggesting that growth factors are stronger inducers of osteocalcin production than mechanostress in osteoblasts.

In the western blot analysis of the immunoprecipitated protein of cultured osteoblasts with exposure to elongation 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, 3B). In this study, we used a hamster anti-gp38 monoclonal antibody derived from clone 8.1.1 (AngioBio) [15,21]. There was no cross reaction except in one higher molecular weight band in the range between 75kDa and 100 kDa. 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. Mouse podoplanin has a 38 kDa protein binding much sialic acid[1,2,3], 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 immunoprecipitated with podoplanin[24]. 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. 3C). These results suggest that mechanostress such as elongation straining induces the production of podoplanin protein. In the matured osteoblast MLO-A5 cell line, an increase of podoplanin is concomitant with dendrite formation, mineralization, and RhoA activation[25]. 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 a RhoA family signaling[26,27]. 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[26,27,28,29]. Therefore, podoplanin may function in the cell shape construction of osteocyte such as dendrite elongation.

There were no statistically significant differences among alive cell numbers of osteoblasts cultured in mineralization medium without any of the antibodies or with anti-podoplanin, anti-osteopontin, anti-osteocalcin, and isotype controls (Fig. 4A), suggesting that the antibodies at the concentration tested here did not affect the cell

viability. 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. However, it has recently been reported that there were no morphological anomalies in the development of podoplanin-deficient alveolar bone or tooth in wingless-related MMTV integration site 1 (*Wnt1*)-*Cre;Pdpn*$^{\Delta/\Delta}$ mice: *Wnt1-Cre* transgenic mice, which express Cre recombinase under the control of *Wnt1* promoter, bred to mice having homozygous podoplanin-floxed alleles *Pdpn*$^{fl/fl}$ [9]. In *Wnt1-Cre;Pdpn*$^{\Delta/\Delta}$ mice the tooth and alveolar grow normally. Therefore, it is thought that podoplanin is not a critical factor in the development of bone. However, these findings may suggest that podoplanin may contribute to the mineralization in osteoblasts under the circumstances of mechanostress in bone with muscle contraction.

V. Conclusion

Mechanostress induces the production of podoplanin in cultured bone marrow-derived osteoblasts, and that podoplanin may contribute to the mineralization in osteoblasts in bone under the circumstances with mechanostress.

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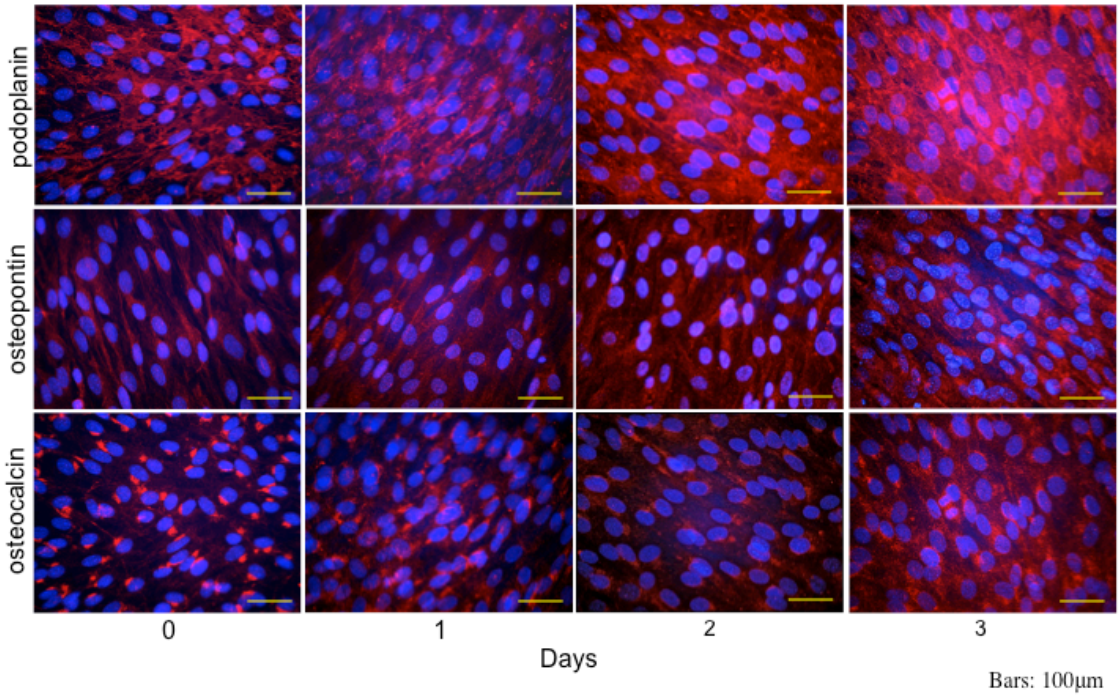

Figure 1

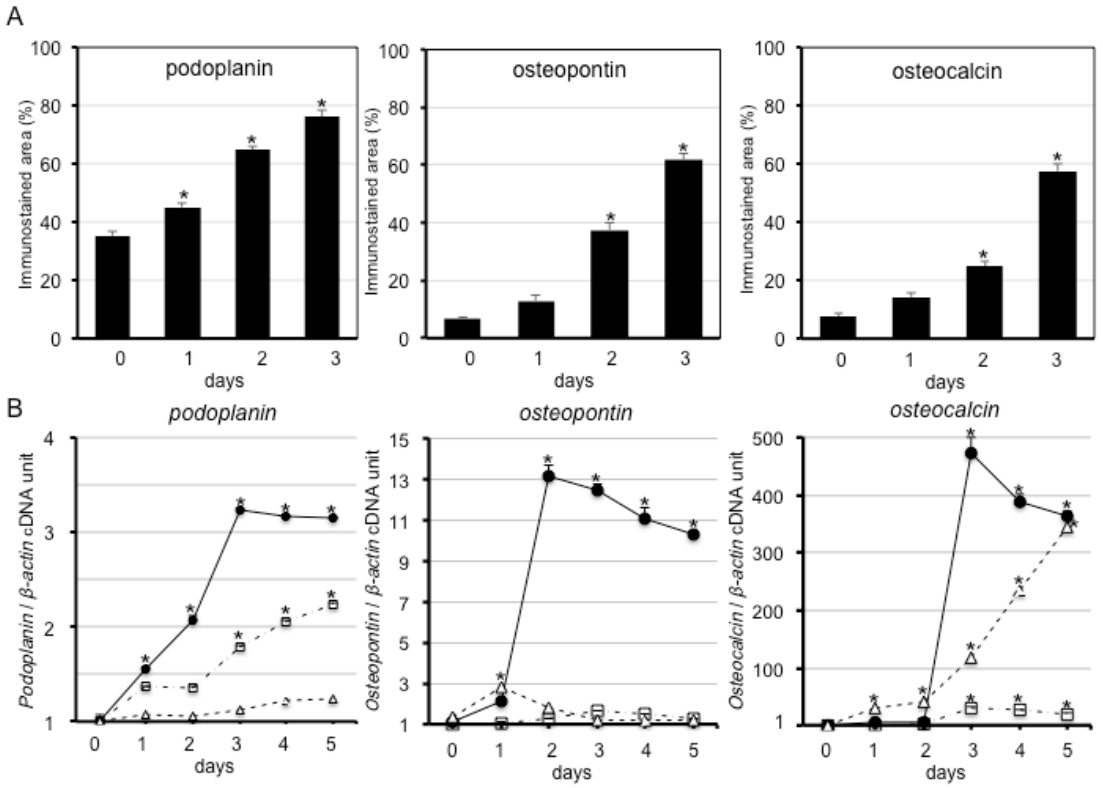
A
podoplanin
osteopontin
osteocalcin
Immunostained area (%)
days
B
podoplanin
osteopontin
osteocalcin
Podoplanin / β-actin cDNA unit
Osteopontin / β-actin cDNA unit
Osteocalcin / β-actin cDNA unit
days

Figure 2

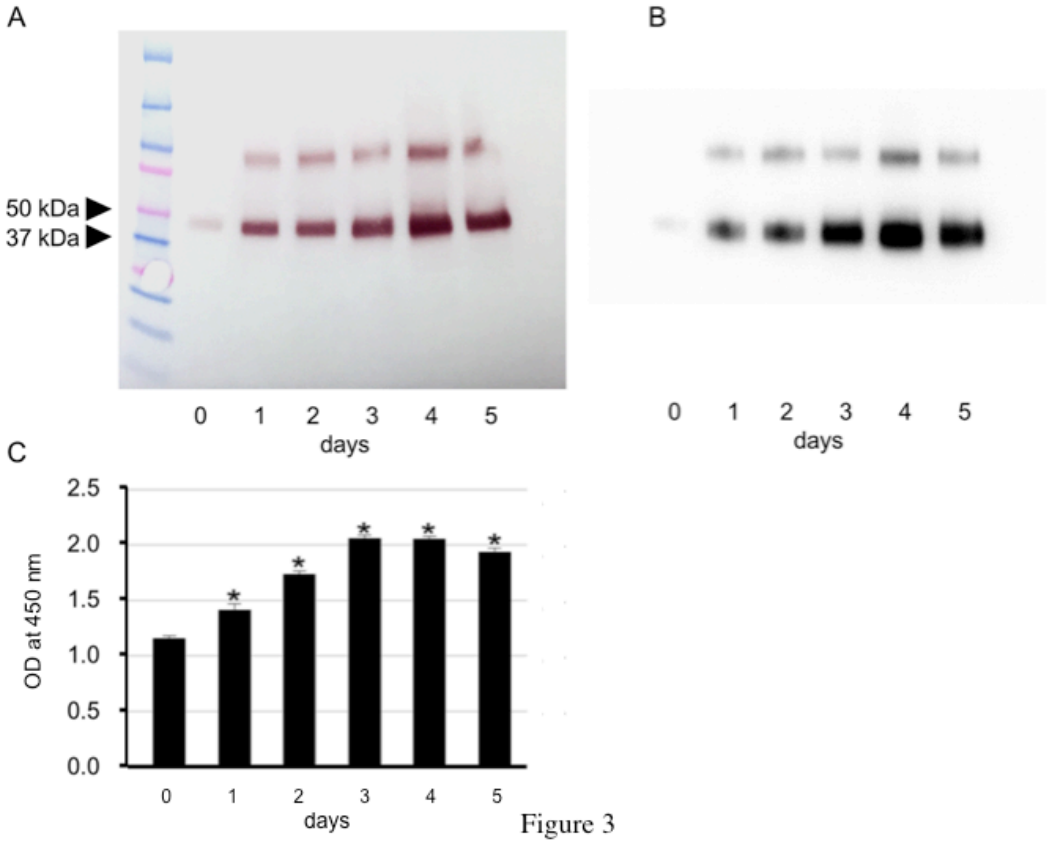
A
50 kDa
37 kDa
0 1 2 3 4 5
days
B
0 1 2 3 4 5
days
C
OD at 450 nm
2.5
2.0
1.5
1.0
0.5
0.0
0 1 2 3 4 5
days

Figure 3

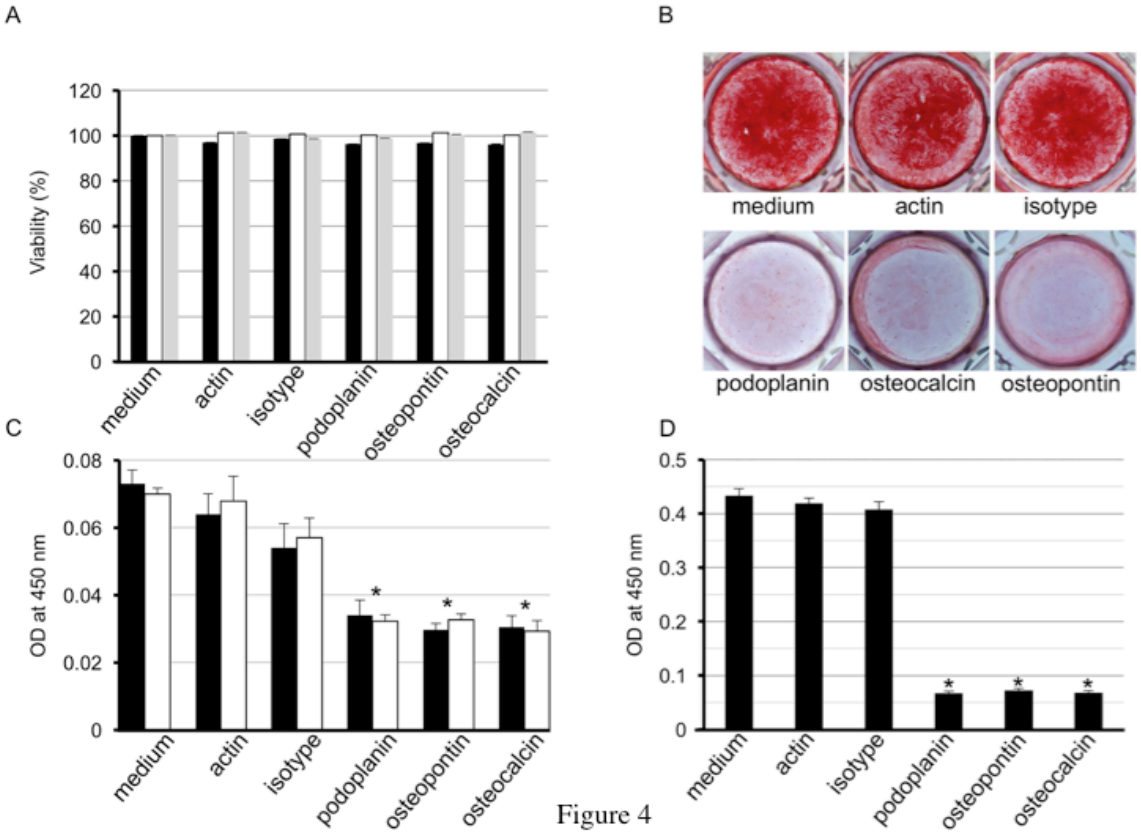
A
B
C
D
Viability (%)
120
100
80
60
40
20
0
medium
actin
isotype
podoplanin
osteopontin
osteocalcin
OD at 450 nm
0.08
0.06
0.04
0.02
0.5
0.4
0.3
0.2
0.1
*

Figure 4

Figure legends

Figure 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 the staining intensity increased with the duration of the elongation straining (days). Nuclei were stained by DAPI. Bars: 100μm.

Figure 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 sectional 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 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 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 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 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 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 larger than in cells cultured with straining in non-mineralization medium. *Significantly different from each 0day control sample in ANOVA ($P<0.01$).

Figure 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 higher MW band between the 75kDa MW and 100 kDa MW markers. **B. The chemiluminescent version of A.** The band increased chemiluminescence density with duration of straining. **C. 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). *Significantly different in ANOVA ($P<0.01$).

Figure 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 ug/ml (open bars) and 2 ug/ml (closed bars) of antibodies for 10 days, and cultured in mineralization medium containing 1 ug/ml of antibodies for 20 days (gray bars). The number of alive cells was estimated by optical density measurements at the 450nm 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 ($P<0.01$). **B. Mineralization assay.** Alizarin red staining was performed on culture products from osteoblasts cultured in the mineralization medium containing 1 ug/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 450nm 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. *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 450nm 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. *Significantly different from medium, anti-actin, and isotype control in ANOVA ($P<0.01$).