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ORIGINAL ARTICLE

Establishment of optimized in vitro assay methods for evaluating osteocyte functions

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Abstract Recent studies have revealed that osteocytes play multiple important physiological roles. To analyze osteocyte functions in detail, an in vitro experimental system for primary osteocytes would be useful. Unfortunately, osteocytes tend to dedifferentiate and acquire osteoblast-like features even when the cells are cultured in three-dimensional (3D) collagen gel. Therefore, it is desirable to establish osteocyte culture conditions that prevent dedifferentiation over longer periods. In this study, we obtained systematic information about the influence of culture conditions on osteocyte differentiation states. Fetal bovine serum (FBS) concentrations from 0.1 to 0.5 % in 3D culture matrix did not significantly influence the expression of osteocyte markers. On the other hand, addition of Matrigel to the culture matrix significantly enhanced the expression of *Rankl* and late osteocyte markers such as *Sost* and *Fgf23*. Matrigel addition also inhibited upregulation of *Opg* and early osteocyte markers such as *Dmp1* and *Gp38*. These effects on osteocyte properties were maximal at a Matrigel culture matrix content of 50 %. Matrigel addition to the matrix also increased dendritic process extension by osteocytes. In addition, Matrigel addition significantly stimulated tartrate-resistant acid phosphatase activity in co-culture with bone marrow macrophages. Among the conditions tested, 50 % Matrigel and 0.2 % FBS in type I collagen matrix were optimal for culture of primary osteocytes.

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Introduction

Osteocytes, which differentiate from osteoblasts encased in bone matrix, acquire stellate cell shapes with dendritic processes that extend through bone canaliculi [2, 16]. Recent progress in understanding the physiological functions of osteocytes has revealed that these cells play central roles in the regulation of both osteoclastic bone resorption and osteoblastic bone formation [19, 23]. Osteocytes secrete sclerostin (SOST), which negatively regulates canonical Wnt pathway signal input into osteoblasts and subsequent osteoblast activation [20, 22]. When osteocytes decrease SOST production in response to external stimuli, suppression of bone formation by nearby osteoblasts is alleviated [17]. Osteocytes also provide a RANKL signal to osteoclast precursors, and thereby regulate bone resorption [8, 19, 23]. Selective deletion of RANKL in osteocytes leads to a significant decrease of osteoclast numbers and suppression of physiological bone remodeling process [23]. Furthermore, osteocytes function as sensors for mechanical stress [1]. Fluid flow in lacunar spaces and deformation of bone both stimulate osteocytes to produce regulatory signals for osteoblasts and osteoclasts [1]. By producing phosphoproteins such as dentin matrix acidic phosphoprotein 1 (Dmp1), osteocytes are also involved in mineral deposition into bone matrix [15]. Osteocytes are involved in not only local but also systemic phosphate homeostasis as a major provider of the phosphaturic factor, fibroblast growth factor (FGF) 23 [3]. To analyze these various osteocyte functions in detail, an in vitro experimental system for osteocytes would be useful.

Isolation of osteocytes requires a great deal of time and effort, and yields are nonetheless low. Consequently, immortalized cell lines such as MLO-Y4 have often been used in previous in vitro analyses [10]. These cell lines have several limitations, however, including the absence of SOST or FGF-23 expression [24], and it is therefore desirable to establish culture conditions and assay methods for primary osteocytes. From this point of view, dedifferentiation of osteocytes during culture is a serious problem that complicates in vitro analyses of osteocyte functions [8, 21]. We previously reported that embedding osteocytes in a collagen gel reduces dedifferentiation during culture, and that such embedded systems can be used to evaluate the osteoclastogenic ability of osteocytes [8]. Even under three-dimensional (3D) culture conditions, however, osteocytes still dedifferentiate over time [8]. To analyze the diverse functions of osteocytes in vitro, it is necessary to develop culture conditions that maintain osteocytic features for longer durations. In this study, we examined the influences of fetal bovine serum (FBS) concentration and addition of Matrigel on the properties of osteocytes in 3D culture. Culture matrix containing Matrigel has often been used in previous studies using MLO-Y4 cells [5, 12, 18]; however; little information is available regarding its effects on osteocytic features. We also evaluated the influence of osteocyte culture conditions on the osteoclastogenic ability of osteocytes.

Materials and methods

Preparation of primary cells and culture conditions

Primary osteocytes were isolated from the calvaria of 1 to 4-day-old C57BL/6 mice, as we described previously [6, 19]. Cells collected from calvaria by enzyme digestion were cultured for 12 h on culture dishes coated with type I collagen (Nitta Gelatin, Osaka, Japan) in α-MEM containing 1 % FBS (Biowest, Nuaillè, France), 20 mM L-glutamine (Nacalai Tesque, Kyoto, Japan), and 1 % penicillin–streptomycin (PCSM, Life Technologies, Carlsbad, CA, USA); adherent cells were used for subsequent experiments. Mouse bone marrow macrophages (BMMs) were collected from the tibia of 8 to 10-week-old C57BL/6 mice using previously reported methods [13]. For the 3D culture system, mouse primary osteocytes were suspended in ice-cold matrix sol [α-MEM containing FBS, L-glutamine, PCSM, Cellmatrix Type I-A (Nitta Gelatin), and Matrigel™ (BD Biosciences, San Jose, CA, USA)]; this mixture was cast into plates and cultured at 37 °C to form collagen hydrogel. To examine the influence of growth factors on osteocyte properties, 50 ng/mL insulin-like growth factor 1 (IGF-1, R&D Systems, Minneapolis, MN, USA), 5 ng/mL transforming growth factor β (TGF-β, R&D Systems), 2.5 ng/mL epidermal growth factor (EGF, R&D Systems) and 25 pg/mL platelet derived growth factor (PDGF, Life Technologies) were added to the matrix sol. All animal procedures were approved by the Institutional Animal Care and Use Committee of Graduate School of Medicine, University of Tokyo. C57BL/6 mice were purchased from Japan SLC (Shizuoka, Japan).

mRNA quantification

Total RNA was extracted using RNAiso Plus (TaKaRa, Shiga, Japan). To extract RNA from osteocytes cultured under 3D conditions, culture matrix containing osteocytes was homogenized in the extraction reagent and processed according to the manufacturer's standard protocol. mRNA expression levels were determined by reverse transcription and real-time quantitative PCR using SYBR® GreenER™ qPCR SuperMix Universal (Life Technologies), an Eco real-time PCR system (Illumina, San Diego, CA, USA), and the associated software. The expression levels of marker genes were calculated using a standard curve prepared by serial dilution of the reference sample. The expression level of each marker gene was normalized to that of a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*). Finally, the relative expression of marker genes in primary osteocytes at each time point was compared with the expression level on day 0. The following primers were used: 5′-CTT CAG GAA TGA TGC CAC AGA GGT-3′ and 5′-ATC TTT GGC GTC ATA GGG ATG GTG-3′ for sclerostin (*Sost*), 5′-ACT TGT CGC AGA AGC ATC-3′ and 5′-GTG GGC GAA CAG TGT ACA A-3′ for fibroblast growth factor 23 (*Fgf23*), 5′-GGC TGT CCT GTG CTC TCC CAG-3′ and 5′-GGT CAC TAT TTG CCT GTC CCT C-3′ for dentin matrix acidic phosphoprotein 1 (*Dmp1*), 5′-CAG TGT TGT TCT GGG TTT TGG-3′ and 5′-TGG GGT CAC AAT ATC ATC TTC A-3′ for podoplanin (*Gp38*), 5′-CCA AGC AGG AGG GCA ATA-3′ and 5′-AGG GCA GCA CAG GTC CTA A-3′ for osteocalcin (*Bglap*), 5′-GGG CGT CTC CAC AGT AAG CG-3′ and 5′-ACT CCC ACT GTG CCC TCG TT-3′ for alkaline phosphatase (*Alpl*), 5′-GTC TGT AGG TAC GCT TCC CG-3′ and 5′-CAT TTG CAC ACC TCA CCA TCA AT-3′ for *Rankl*, 5′-ACC CAG AAA CTG GTC ATC AGC-3′ and 5′-CTG CAA TAC ACA CAC TCA TCA CT-3′ for *Opg*, and 5′-ATG TGT CCG TCG TGG ATC TG-3′ and 5′-TGA AGT CGC AGG AGA CAA CC-3′ for *Gapdh*.

Quantification of secreted proteins using ELISA

Medium was collected every other day (days 1, 3, 5, 7) and replaced with fresh medium. We measured the

concentration of substances in the collected medium, and the results are expressed as accumulated values. The concentrations of the soluble forms of RANKL (sRANKL), OPG, SOST, and FGF23 in culture supernatants were measured using the Mouse TRANCE/RANKL/TNFSF11 Quantikine® ELISA Kit (R&D Systems), Mouse Osteoprotegerin/TNFRSF11B Quantikine® ELISA Kit (R&D Systems), Mouse/Rat SOST Quantikine ELISA Kit (R&D Systems), and FGF-23 ELISA Kit (KAINOS Laboratories, Tokyo, Japan), respectively, following the manufacturer's protocols.

Construction of protein expression vectors

To introduce green fluorescent protein (GFP) into osteocytes, a lentiviral expression vector was constructed and used as we previously described [8].

Cell staining and confocal laser scanning microscopy analysis

For actin fiber staining, cultured cells were treated with 4 % paraformaldehyde and 0.02 % Triton-X100, and phalloidin tetramethylrhodamine B isothiocyanate (TRITC) conjugate (Sigma-Aldrich, St. Louis, MO, USA) was used according to the manufacturer's protocol. For visualization of intercellular connections, cultured cells were incubated for 30 min with 5 μM Cellstain Calcein-AM (DOJINDO Laboratories, Kumamoto, Japan) and 20 μM Hoechst 33342 (Sigma-Aldrich). Fluorescence was detected using a Fluoview FV1000 confocal laser scanning microscope (Olympus, Tokyo, Japan) or a TCS SP8 confocal laser scanning microscope (Leica Microsystems, Wetzler, Germany). Three-dimensional reconstructions of confocal sectioning images and filament tracer analyses were performed using the IMARIS software (Bitplane, Zurich, Switzerland).

Co-culture assay with bone marrow macrophages

A 3D co-culture of osteocytes with BMMs was performed as we described previously [8]. First, osteocytes were cultured for 8 h on membranes with 3 μm diameter pores (Millipore, Bedford, MA, USA) with collagen coating. Subsequently, matrix sol was cast into culture plates, and the porous membranes with osteocytes attached on one side were placed on the surface to embed the osteocytes in matrix sol. After 1 h of incubation at 37 °C to gelatinize matrix sol, BMMs suspended in α-MEM containing 10 % FBS, L-glutamine, PCSM, and 50 ng/mL mouse macrophage colony-stimulating factor (M-CSF, R&D Systems) were seeded on top of the membranes. The culture medium was replaced with fresh medium every 3 days. Tartrate-resistant acid phosphatase (TRAP) enzymatic activity, which was evaluated by measuring *p*-nitrophenol production from *p*-nitrophenyl phosphate in the presence of tartaric acid, was assessed after 7 days of co-culture, using the TRAP and ALP assay kit (TaKaRa) following the manufacturer's protocol.

Statistical analysis

All data are expressed as mean ± SD from three independent determinations, except where noted. Statistical analysis was performed using Student's *t* test or analysis of variance (ANOVA), followed by Dunnett's test where applicable.

Results

FBS concentration between 0.1 and 0.5 % has little impact on osteocyte properties in 3D culture

Previously, we reported that type I collagen gel containing 1.0 % FBS can be used in a 3D osteocyte culture system [8]; however, the influence of FBS content on osteocyte properties has not been extensively evaluated. To address this issue, we embedded isolated mouse osteocytes in collagen gel containing 0.1, 0.2, 0.5, or 1.0 % FBS, and then extracted total RNA at the indicated time points. Using a real-time PCR method, we then quantitated mRNA expression levels of osteocyte and osteoblast marker genes (Fig. 1a). The results showed that mRNA expression levels of the mature osteocyte markers *Sost* and *Fgf23* tended to decrease after day 7 under 3D culture conditions, and that the magnitude of the decrease was slightly smaller in 0.1 and 0.2 % FBS than in 1.0 % FBS. Similarly, mRNA expression levels of *Dmp1* and *Gp38*, early osteocyte markers, increased at day 9 under 3D culture conditions, and the magnitude of the increase was slightly smaller in 0.1–0.5 % FBS than in 1.0 %. We also measured the expression levels of two osteoblast markers, *Bglap* and *Alpl*; the expression levels of these genes were essentially stable under 3D culture conditions. In addition, we measured the mRNA expression levels of *Rankl* and *Opg*. *Rankl* tended to be downregulated, and *Opg* tended to be upregulated, after day 7 under 3D culture conditions. The magnitude of the decrease in *Rankl* expression was slightly smaller in 0.1–0.2 % FBS than in 1.0 % FBS; by contrast, FBS concentration did not affect *Opg* expression level. Subsequently, we quantitated secreted SOST, FGF23, sRANKL, and OPG in the culture medium using ELISA (Fig. 1b). The SOST secretion rate gradually declined with time under 3D culture conditions, but it remained slightly higher in 0.1–0.5 % FBS than in 1.0 % FBS. Secretion rates of FGF23 and sRANKL, which gradually declined over time under 3D culture

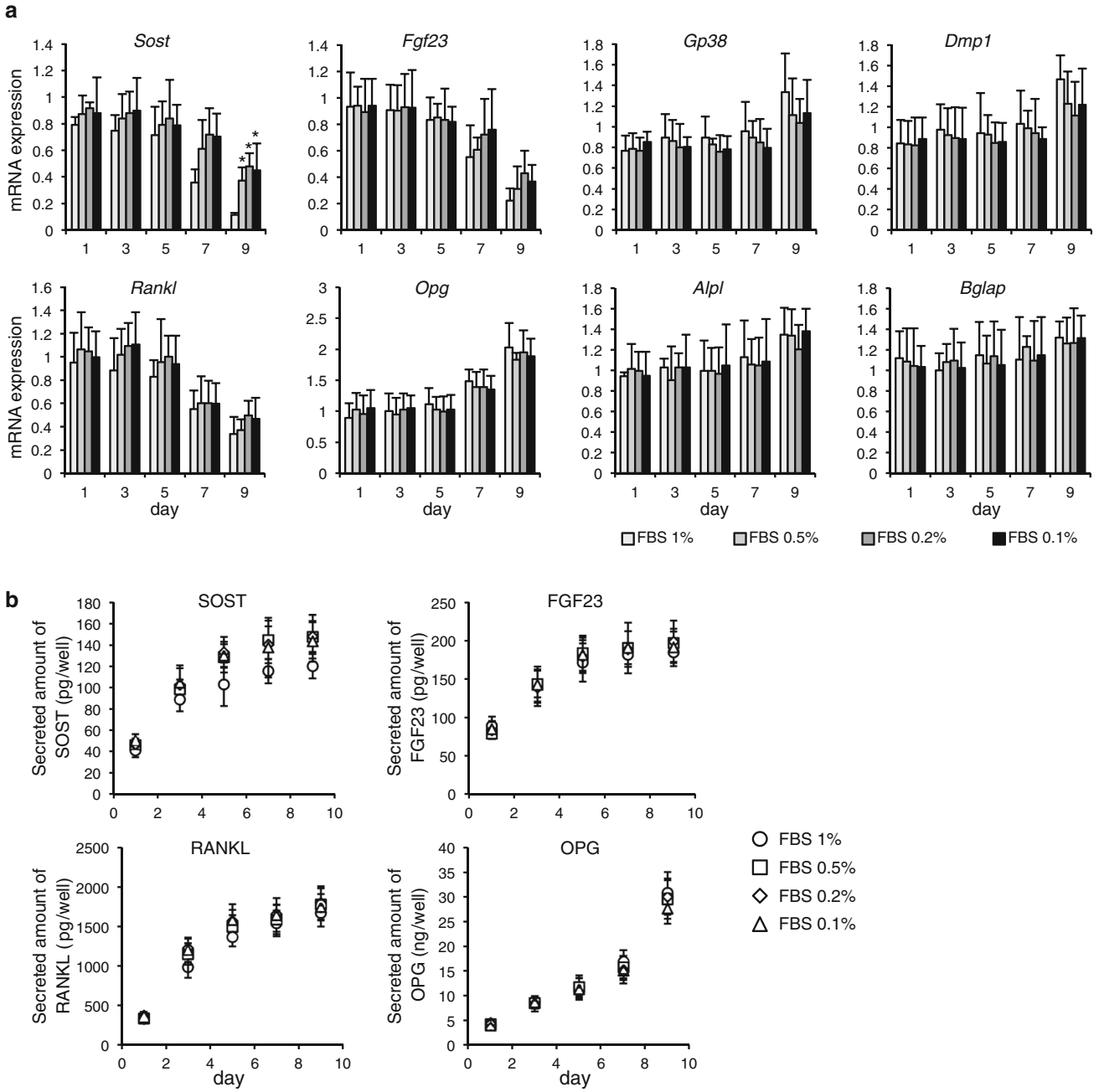


Fig. 1 FBS concentration in culture matrix has little impact on osteocyte differentiation states. **a** Effect of FBS concentration on mRNA expression levels of marker genes in primary osteocytes. Freshly isolated osteocytes were cultured in collagen gel containing FBS at the indicated concentrations, and total RNA samples were collected at the indicated times. The relative expression level of each marker gene at each time point was compared with that on day 0 (freshly isolated cells). $n = 3$, $^*P < 0.05$ vs. FBS 1.0 %. **b** Effect of FBS concentration on marker protein secretion from primary osteocytes. The concentration of each marker protein was quantitated using ELISA, and the cumulative amount of secreted protein is shown. $n = 3$

conditions, were not affected by FBS concentrations. Similarly, FBS concentration did not affect the secretion rate of OPG, which tended to increase after day 7. These results indicated that differences in FBS concentration within the range 0.1–0.5 % have little impact on the differentiation states of osteocytes cultured in collagen gel.

Osteocyte properties are maintained for longer durations when Matrigel is added to collagen gel

Some previous studies have used Matrigel for MLO-Y4 osteocytic cell culture, and the results suggested that Matrigel promotes dendritic process formation in 3D cultures

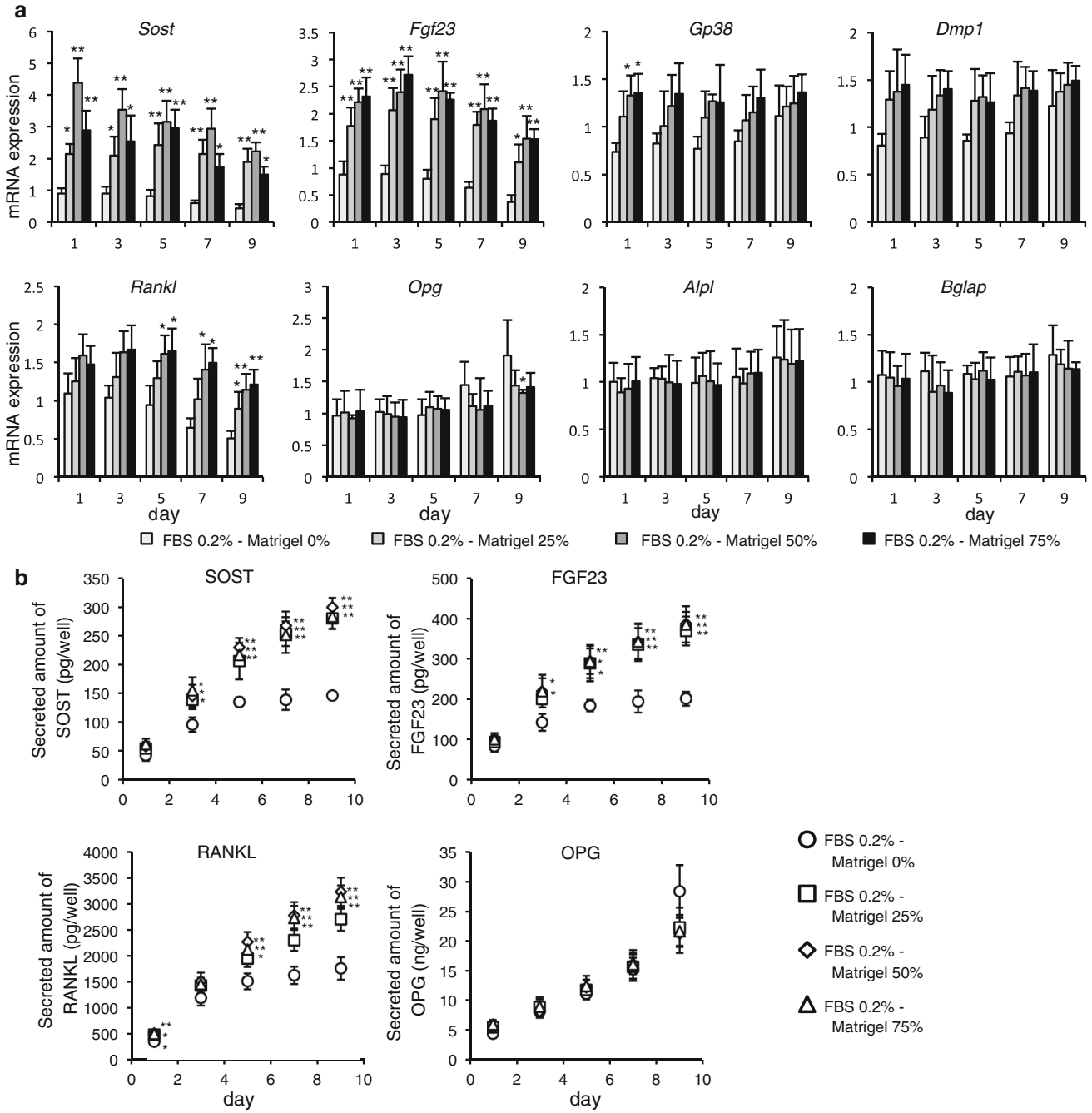


Fig. 2 Osteocyte features are promoted by addition of Matrigel to the culture matrix. **a** Effect of Matrigel addition on mRNA expression levels of marker genes in primary osteocytes. Freshly isolated osteocytes were cultured in collagen gel containing Matrigel at the indicated concentrations, and total RNA samples were collected at the indicated times. FBS concentration was fixed at 0.2 %. The relative expression level of each marker gene at each time point was compared with that on day 0 (freshly isolated cells). $n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$ vs. Matrigel 0 %. **b** Effect of Matrigel addition on marker protein secretion from primary osteocytes. The concentration of each marker protein was quantitated using ELISA, and the cumulative amount of secreted protein is shown. $n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$ vs. Matrigel 0 %

[5, 12]; however, the effects of Matrigel on the properties of primary osteocytes remain to be elucidated. To address this issue, we embedded freshly isolated mouse osteocytes in collagen gel containing 25, 50, or 75 % Matrigel; in these experiments, FBS concentration in the culture matrix was fixed at 0.2 %. Total RNA was extracted at the indicated time points, and mRNA expression levels of osteocyte and osteoblast markers were quantitated using real-time PCR (Fig. 2a). The results showed that mRNA expression levels of *Sost* and *Fgf23* were significantly

upregulated by Matrigel addition, and this effect was maximal at a Matrigel concentration of 50 %. Similarly, mRNA expression levels of *Dmp1* and *Gp38* were also upregulated by Matrigel addition, and this effect was also maximal at 50 %. The expression levels of the osteoblast markers *Bglap* and *Alpl* were not affected by Matrigel addition. Measurement of *Rankl* mRNA expression revealed that its profile was similar to those of *Sost* and *Fgf23*: *Rankl* expression was upregulated by Matrigel addition, and the effect was maximal at 50 %. By contrast, the increase in *Opg* expression after day 7 was suppressed by addition of Matrigel, and this effect was maximal at 50 %. We also quantitated the levels of the marker proteins SOST, FGF23, sRANKL, and OPG in the culture medium, using ELISA (Fig. 2b). The results showed that SOST, FGF23, and sRANKL secretion rates were significantly upregulated, and stayed relatively stable during culture in the presence of Matrigel. On the other hand, the increase in the OPG secretion rate after day 7 was suppressed by Matrigel addition. These results suggested that addition of Matrigel increases the duration that osteocyte differentiation states can be maintained in 3D culture.

Matrigel addition leads to the extension of osteocyte dendritic processes

We also examined the effects of Matrigel on osteocyte morphologic properties. To visualize cell shapes, isolated osteocytes were transduced with GFP before being embedded in matrix, and the cells were stained with TRITC-phalloidin to visualize actin fiber orientation. Osteocytes extended dendritic processes in various directions three-dimensionally, so we obtained *z* stack fluorescence images of osteocytes using confocal laser scanning microscopy, and projected the reconstructed 3D images onto the *x*–*y* plane (Fig. 3a). Stellate shapes with cell processes were observed in both cases, but the length of dendritic processes was longer in matrix containing 50 % Matrigel. To confirm this point quantitatively, we performed image analysis. Each reconstructed 3D image was analyzed using the IMARIS software to measure the length of dendritic processes, as depicted in Fig. 3b. The results showed that the number of dendritic processes longer than 15 μm was significantly higher in osteocytes cultured in matrix containing Matrigel (Fig. 3c). The average length and maximum length of the dendritic processes were also higher in the presence of Matrigel (Fig. 3c). We also obtained low-magnification images of a number of cells to examine cell–cell interactions between osteocytes via their dendritic processes; for these analyses, cultured osteocytes were stained with calcein-AM to visualize the cell shapes, and with Hoechst 33342 to visualize osteocyte nuclei. Intercellular connections formed when Matrigel was added to the 3D culture matrix (Fig. 3d). Together, these results demonstrate that the addition of Matrigel to 3D culture matrix promotes the maintenance of osteocyte morphologic properties.

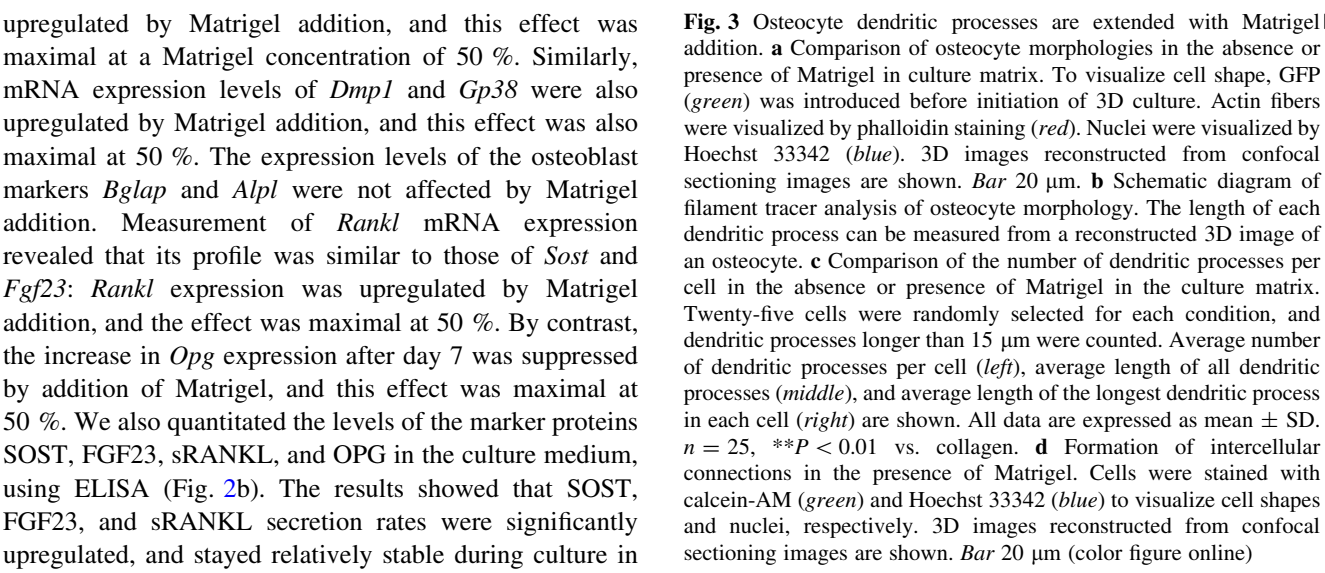
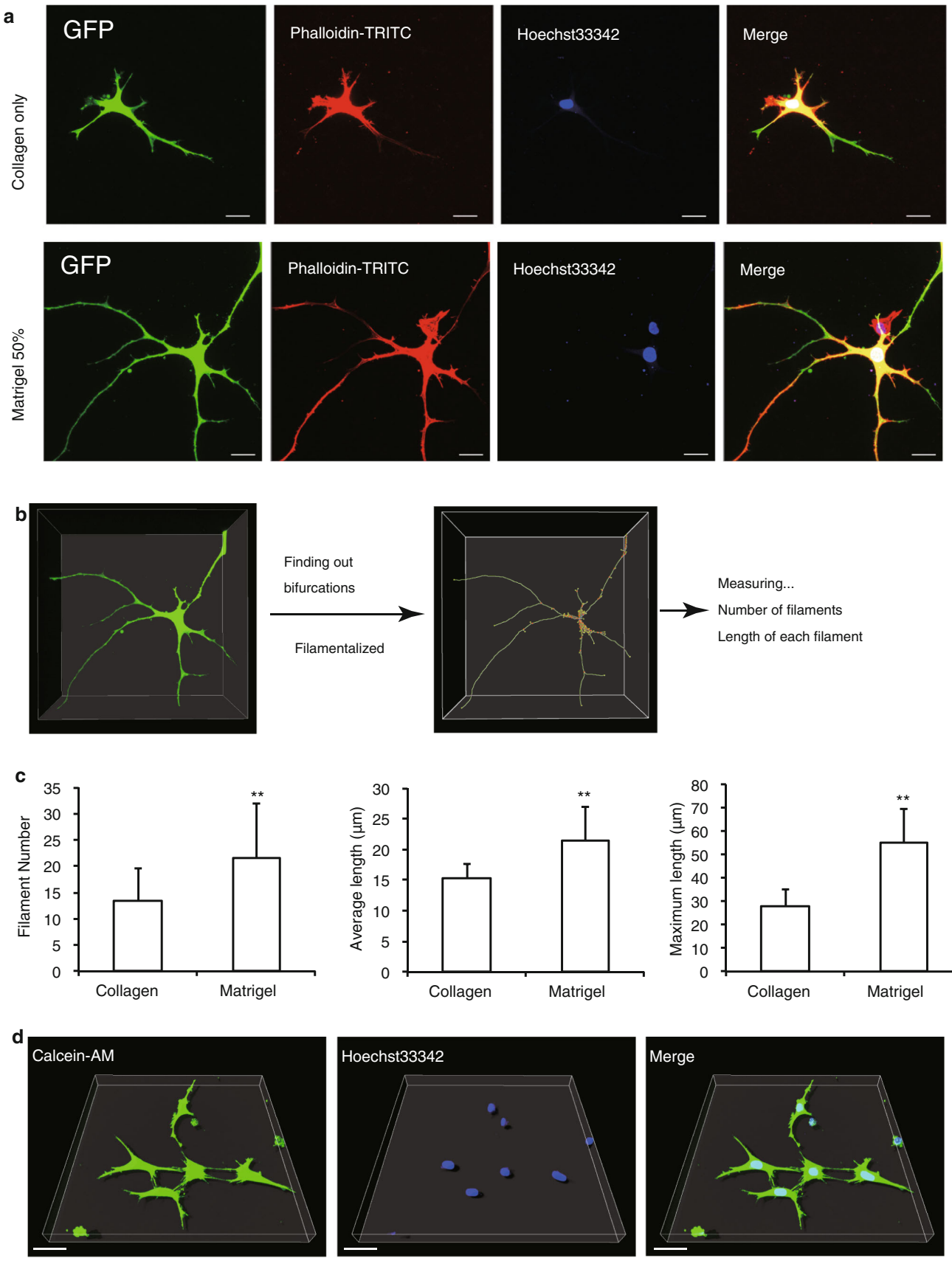


Fig. 3 Osteocyte dendritic processes are extended with Matrigel addition. **a** Comparison of osteocyte morphologies in the absence or presence of Matrigel in culture matrix. To visualize cell shape, GFP (*green*) was introduced before initiation of 3D culture. Actin fibers were visualized by phalloidin staining (*red*). Nuclei were visualized by Hoechst 33342 (*blue*). 3D images reconstructed from confocal sectioning images are shown. *Bar* 20 μm. **b** Schematic diagram of filament tracer analysis of osteocyte morphology. The length of each dendritic process can be measured from a reconstructed 3D image of an osteocyte. **c** Comparison of the number of dendritic processes per cell in the absence or presence of Matrigel in the culture matrix. Twenty-five cells were randomly selected for each condition, and dendritic processes longer than 15 μm were counted. Average number of dendritic processes per cell (*left*), average length of all dendritic processes (*middle*), and average length of the longest dendritic process in each cell (*right*) are shown. All data are expressed as mean ± SD. $n = 25$, $^{**}P < 0.01$ vs. collagen. **d** Formation of intercellular connections in the presence of Matrigel. Cells were stained with calcein-AM (*green*) and Hoechst 33342 (*blue*) to visualize cell shapes and nuclei, respectively. 3D images reconstructed from confocal sectioning images are shown. *Bar* 20 μm (color figure online)

Matrigel effects on the maintenance of osteocyte properties cannot be attributed to basement membrane components or several growth factors contained in Matrigel

Matrigel, a protein mixture secreted from Engelbreth-Holm-Swarm mouse sarcoma cells, contains various growth factors, as well as basement membrane components [11]. To eliminate the influence of growth factors, growth factor–reduced (GFR) Matrigel is available from the manufacturer. Hence, we compared the effects of GFR and conventional Matrigel on marker gene expression levels in osteocytes (Fig. 4a). The increases in Sost, Fgf23, and Rankl mRNA expression levels in osteocytes were significantly lower when examined using the GFR Matrigel than when examined using conventional Matrigel. Similarly, GFR Matrigel had little effect on mRNA expression levels of *Dmp1*, *Gp38, Bglap, Alpl,* and *Opg*. These results indicated that Matrigel constituents other than basement membrane components are responsible for the effect of conventional Matrigel on the maintenance of osteocyte properties. Considering this, we next examined the influence of IGF-1, TGF-β, EGF, and PDGF, all of which are components of Matrigel, on osteocyte differentiation status in 3D cultures with collagen-only gel (Fig. 4b). The stimulatory effects of growth factors on *Sost* and *Fgf23* mRNA expression levels in osteocytes were significantly weaker than those of Matrigel. Similarly, addition of growth factors exerted little or no effect on mRNA expression levels of *Dmp1*, *Gp38*, *Bglap*, *Alpl*, *Rankl*, and *Opg*. We also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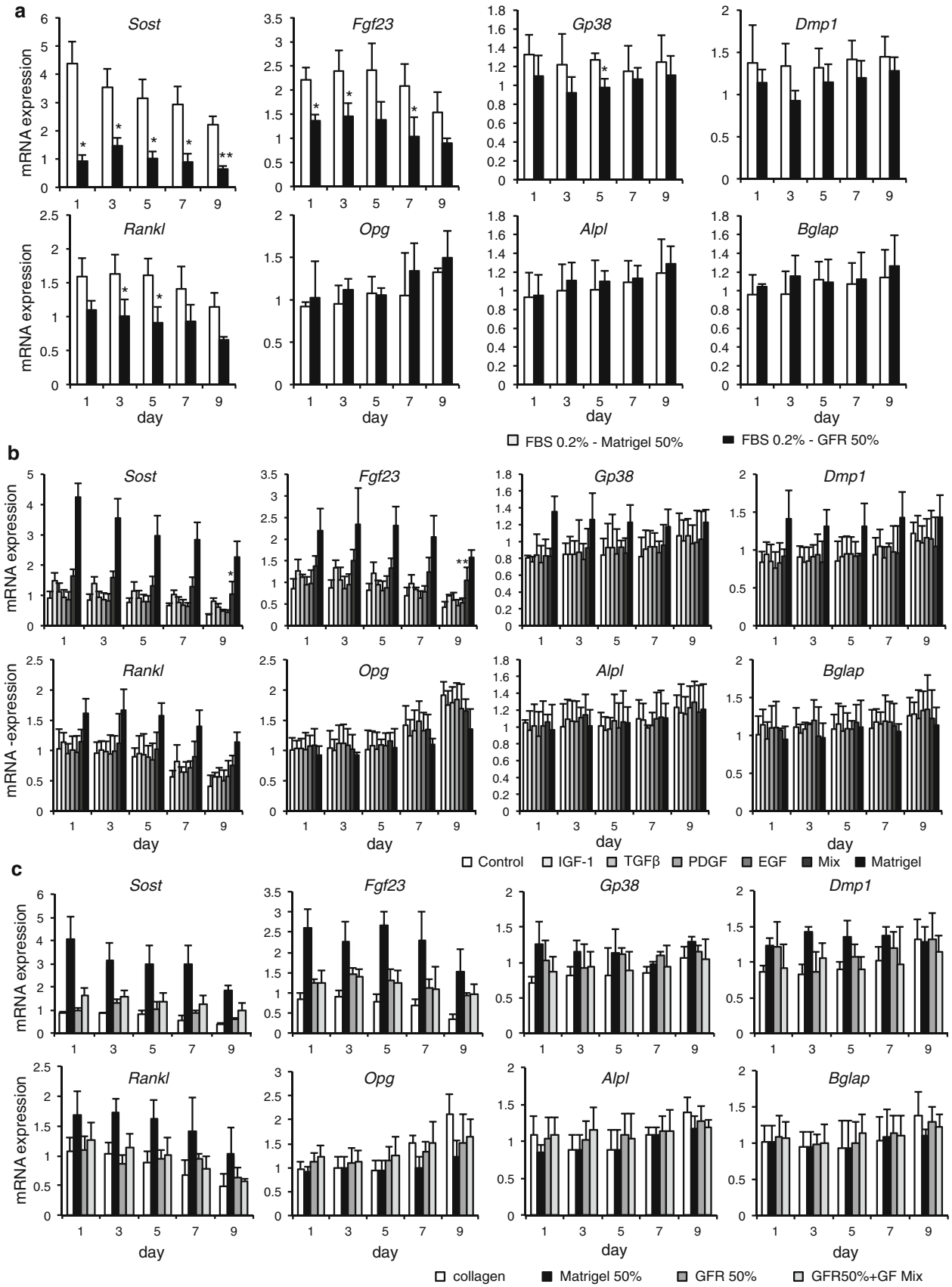
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◀**Fig. 4** Effect of Matrigel on osteocyte differentiation states is neither due to basement membrane components nor major constituent growth factors. **a** Comparison of the effects of conventional and GFR Matrigel on mRNA expression levels of marker genes in primary osteocytes. Freshly isolated osteocytes were cultured in 3D matrix, and total RNA samples were collected at the indicated times. The relative expression level of each marker gene at each time point was compared with that on day 0 (freshly isolated cells). $n = 3$, $*P < 0.05$, $**P < 0.01$ vs. conventional Matrigel. **b** Effect of growth factors (IGF-1, TGF-β, EGF, and PDGF) on mRNA expression levels of marker genes in primary osteocytes cultured in collagen gel. Freshly isolated osteocytes were cultured in collagen gel containing growth factors, and total RNA samples were collected at the indicated times. The relative expression level of each marker gene at each time point was compared with that on day 0 (freshly isolated cells). $n = 3$, $*P < 0.05$, $**P < 0.01$ vs. control. **c** Effect of growth factors on mRNA expression levels of marker genes in primary osteocytes cultured in 50 % GFR Matrigel. Freshly isolated osteocytes were cultured in 50 % GFR Matrigel containing growth factors, and total RNA samples were collected at the indicated times. The relative expression level of each marker gene at each time point was compared with that on day 0 (freshly isolated cells). $n = 3$, $*P < 0.05$, $**P < 0.01$ vs. control

examined whether growth factors added to 50 % GFR Matrigel had any effect on the expression profiles of osteocyte markers (Fig. 4c). The results showed that adding these growth factors to GFR Matrigel did not reproduce the effects on osteocyte properties mediated by conventional Matrigel. Therefore, these growth factors are not responsible for the effects of conventional Matrigel on the maintenance of osteocyte differentiation states.

Addition of Matrigel to 3D culture matrix increased osteocyte ability to stimulate TRAP activity in a co-culture system

Previously, we described a co-culture system that enables evaluation of the ability of osteocytes to support osteoclastogenesis [8]. This system consists of a block of 3D culture matrix containing embedded osteocytes, upon which BMMs are seeded (Fig. 5a). In this co-culture assay system, the two cell types are separated by a porous membrane; therefore, direct interactions between osteocytes and BMMs are only allowed at the extremities of osteocyte dendritic processes which extend through membrane pores. Using this assay system, we evaluated the effect of matrix components on the ability of osteocytes to stimulate TRAP activity. The results showed that the influence of FBS concentration on osteocyte osteoclastogenic ability was minor (Fig. 5b). On the other hand, the addition of Matrigel to 3D culture matrix significantly enhanced TRAP activity in the co-culture system (Fig. 5c), and this effect was maximal at 50 % Matrigel. These

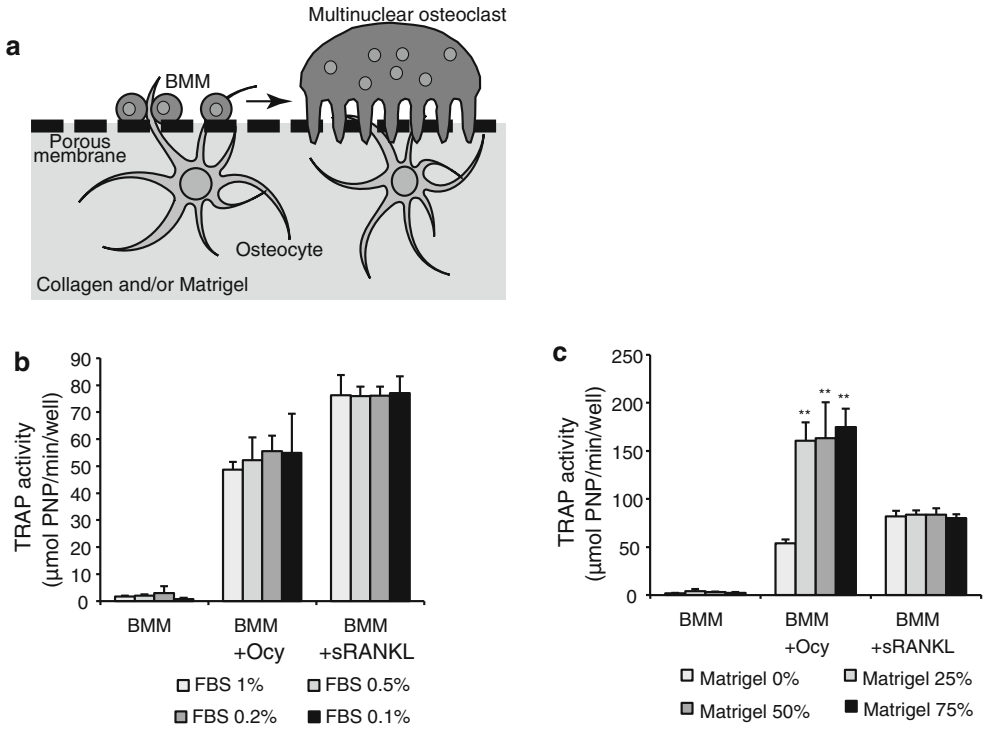

Fig. 5 Addition of Matrigel to the culture matrix significantly increased TRAP activity in co-culture of osteocytes and BMMs. **a** Schematic diagram of osteocytes co-cultured with BMMs to examine the osteoclastogenic ability of osteocytes. **b** Influence of FBS concentration in the culture matrix on TRAP activity in a co-culture of BMMs and osteocytes, prepared as depicted in **a**. $n = 3$. **c** The effect of Matrigel in the culture matrix on TRAP activity in a co-culture of BMMs and osteocytes. $n = 3$, $**P < 0.01$ vs. Matrigel 0 %

results are consistent with the observations, described above, that mRNA expression was upregulated in osteocytes cultured in matrix containing Matrigel.

Discussion

Osteocytes tend to dedifferentiate and acquire osteoblast-like features during in vitro culture after isolation [8, 21], making it difficult to analyze osteocyte functions in detail. Therefore, it would be useful to improve the conditions used for culture of osteocytes. We have previously reported that the osteoclastogenic ability of osteocytes can be evaluated using a 3D culture system in type I collagen gel containing 1.0 % FBS [8]. Unfortunately, osteocytes dedifferentiate over time, even under 3D culture conditions in type I collagen. To enable further analyses of the physiological properties of osteocytes, it is desirable to optimize the culture conditions for primary osteocytes. To date, the available information about the influence of culture conditions on osteocytic features has been insufficient. The majority of reports describing culture condition for osteocytes have evaluated the influence of various factors on osteocyte morphologies [5, 12, 18], whereas the influence of these factors on osteocytic marker expression profiles remains to be examined. In this study, we obtained systematic information about the influence of culture conditions on various osteocyte properties.

First, we confirmed that FBS content between 0.1 and 1.0 % in collagen gel does not have a large impact on the marker gene expression profiles of osteocytes. Complete depletion of FBS from the culture matrix and medium resulted in morphological aberrations in cultured osteocytes, probably due to a reduction in cell viability (data not shown). Based on these results, we judged that 0.2 % FBS concentration is preferable for 3D culture of osteocytes. Next, we examined the effect of addition of Matrigel to the culture matrix. Previous reports showed that a mixture of type I collagen and Matrigel permits the MLO-Y4 osteocytic cell line to maintain dendritic morphology and intercellular connectivity [5, 12, 18]. Other reports have shown that Matrigel promotes the differentiation of osteoblastic cells, such as MC-3T3E1, and promotes their acquisition of osteocytic morphologies [9]. However, the influence of Matrigel on other properties of osteocytes has been unclear. To address this issue, we examined the influence of Matrigel on the expression of various osteocytic marker genes. Addition of Matrigel affected not only the morphologies of primary osteocytes, but also the expression levels of various osteocytic marker genes. These effects were maximal when the content of Matrigel in the culture matrix was 50 %. The expression of mRNA for *Sost* and *Fgf23* (late osteocyte markers) was upregulated more than twofold after the start of 3D culture in the Matrigel-containing matrix when compared with that in freshly isolated cell population. Similarly, the expression of mRNA for *Gp38* (early osteocyte marker) was also upregulated significantly upon embedding in Matrigel-containing matrix. We previously reported that the primary osteocyte-rich population, prepared as described in the present study, contains a certain proportion of early osteocytes and osteoblasts [8]. If we assume that a certain proportion of early osteocytes differentiated into mature osteocytes upon embedding in the 3D matrix, this may explain the increase in the expression levels of late osteocyte markers. Similarly, if the contribution of the increase in *Gp38* mRNA that occurs during the differentiation of osteoblasts to early osteocytes upon embedding in Matrigel-containing matrix is greater than the contribution of the decrease in *Gp38* mRNA that accompanies the differentiation of early osteocytes to late osteocytes, the total amount of *Gp38* mRNA expressed in the cell population will be higher than that in freshly isolated cell population. On the other hand, the expression levels of late osteocyte markers tended to decrease over time, even in cells cultured in the Matrigel-containing matrix, suggesting that Matrigel does not completely inhibit the dedifferentiation of mature osteocytes. The expression level of *Gp38* tended to increase slightly during culture especially in collagen-only matrix, also suggesting a possibility that a certain proportion of mature osteocytes dedifferentiate to early osteocytes during culture. Even so, the expression of mRNA for late osteocyte markers was comparable, or higher, than that in the freshly isolated cell population at day 9. On the other hand, the results of the present study show that the expression of osteoblast markers by cells in the 3D Matrigel matrix-containing culture system was stable. It is reported that the expression of osteoblast markers such as *Alpl* and *Bglap* tends to decrease as osteoblasts differentiate into early osteocytes [2]. We previously reported that expression of mRNA for osteoblast markers by primary osteocytes in the 2D culture system tends to increase over time [8]. Considering these, we can assume that the dedifferentiation of early osteocytes into more immature osteoblastic cells, which takes place under 2D culture conditions, is inhibited under 3D culture conditions. Based on all of these observations, we conclude that 50 % Matrigel and 0.2 % FBS in type I collagen matrix is an optimal condition for culturing primary osteocytes.

Next, we tried to identify the Matrigel components that contribute to maintenance of osteocytic differentiation states. Matrigel is a reconstituted basement membrane preparation whose major constituents are laminin, collagen type IV, entactin, and perlecan. Previous report showed that the addition of Matrigel increased matrix stiffness when added to type-I collagen matrix [4]. However, our

results indicated that these components make only a limited contribution to the maintenance of osteocytic properties. We also examined the contribution of growth factors contained in Matrigel. We selected IGF-1, TGF-β, EGF, and PDGF as candidates, because the manufacturer has shown that the levels of these growth factors are significantly reduced in GFR Matrigel relative to conventional Matrigel, or these growth factors are present in physiological bone matrix [7, 14]. The results indicated that these candidate growth factors, either alone or in combination, do not explain the entire effect of Matrigel to maintain osteocyte differentiation states. Based on these observations, we hypothesize that other growth factors contained in Matrigel are responsible for the maintenance of osteocyte properties. Although the involvement of major growth factors known to be contained in Matrigel were examined in this study, it is possible that Matrigel contains other growth factors secreted from EHS sarcoma cells at significant levels. To advance our understanding of the effects of Matrigel on osteocytes further, it will be necessary to analyze Matrigel contents in more detail.

Finally, we evaluated the utility of this 3D osteocyte culture system for in vitro analyses of osteocyte physiological functions. Matrigel addition to the culture matrix of osteocytes significantly enhanced TRAP activity in the co-cultures. This observation is consistent with results showing that Matrigel addition delayed downregulation of RANKL expression and upregulation of OPG expression in cultured osteocytes. This improved system for co-culture of osteocytes with BMMs will enable detailed analyses of the molecular mechanisms involved in the osteoclastogenic process.

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Conflict of interest All authors state that they have no conflict of interest.

References

1. Bonewald LF, Johnson ML (2008) Osteocytes, mechanosensing and Wnt signaling. Bone 42:606–615
2. Bonewald LF (2011) The amazing osteocyte. J Bone Miner Res 26:229–238
3. Cheng F, Hulley P (2010) The osteocyte—a novel endocrine regulator of body phosphate homeostasis. Maturitas 67:327–338
4. Dewitt DD, Kaszuba SN, Thompson DM, Stegemann JP (2009) Collagen I-Matrigel scaffolds for enhanced Schwann cell survival and control of three-dimensional cell morphology. Tissue Eng Part A 15:2785–2793
5. Fukunaga T, Kurata K, Matsuda J, Higaki H (2008) Effects of strain magnitude on mechanical responses of three-dimensional gel-embedded osteocytes studied with a novel 10-well elastic chamber. J Biomech Sci Eng 3:13–24
6. Gu G, Nars M, Hentunen TA, Metsikko K, Vaananen HK (2006) Isolated primary osteocytes express functional gap junctions in vitro. Cell Tissue Res 323:263–271
7. Hauschka PV, Chen TL, Mavrakos AE (1988) Polypeptide growth factors in bone matrix. Ciba Found Symp 136:207–225
8. Honma M, Ikebuchi Y, Kariya Y, Hayashi M, Hayashi N, Aoki S et al (2013) RANKL subcellular trafficking and regulatory mechanisms in osteocytes. J Bone Miner Res 28:1936–1949
9. Karsdal MA, Larsen L, Engsig MT, Lou H, Ferreras M, Lochter A et al (2002) Matrix metalloproteinase-dependent activation of latent transforming growth factor-β controls the conversion of osteoblasts into osteocytes by blocking osteoblast apoptosis. J Biol Chem 277:44061–44067
10. Kato Y, Windle JJ, Koop BA, Mundy GR, Bonewald LF (1997) Establishment of an osteocyte-like cell line, MLO-Y4. J Bone Miner Res 12:2014–2023
11. Kleinman HK, McGarvey ML, Liotta LA, Robey PG, Tryggvason K, Martin GR (1982) Isolation and characterization of type IV procollagen, laminin, and heparan sulfate proteoglycan from the EHS sarcoma. Biochemistry 21:6188–6193
12. Kurata K, Fukunaga T, Matsuda J, Higaki H (2007) Role of mechanically damaged osteocytes in the initial phase of bone remodeling. Int J Fatigue 29:1010–1018
13. Li X, Udagawa N, Takami M, Sato N, Kobayashi Y, Takahashi N (2003) p38 Mitogen-activated protein kinase is crucially involved in osteoclast differentiation but not in cytokine production, phagocytosis, or dendritic cell differentiation of bone marrow macrophages. Endocrinology 144:4999–5005
14. Lind M (1996) Growth factors: possible new clinical tools: a review. Acta Orthop 67:407–417
15. Liu S, Gupta A, Quarles LD (2007) Emerging role of fibroblast growth factor 23 in a bone-kidney axis regulating systemic phosphate homeostasis and extracellular matrix mineralization. Curr Opin Nephrol Hypertens 16:329–335
16. McNamara LM, Majeska RJ, Weinbaum S, Friedrich V, Schaffler MB (2009) Attachment of osteocyte cell processes to the bone matrix. Anat Rec (Hoboken) 292:355–363
17. Moester MJ, Papapoulos SE, Lowik CW, van Bezooijen RL (2010) Sclerostin: current knowledge and future perspectives. Calcif Tissue Int 87:99–107
18. Mulcahy LE, Taylor D, Lee TC, Duffy GP (2011) RANKL and OPG activity is regulated by injury size in networks of osteocyte-like cells. Bone 48:182–188
19. Nakashima T, Hayashi M, Fukunaga T, Kurata K, Oh-Hora M, Feng JQ et al (2011) Evidence for osteocyte regulation of bone homeostasis through RANKL expression. Nat Med 17:1231–1234
20. ten Dijke P, Krause C, de Gorter DJ, Lowik CW, van Bezooijen RL (2008) Osteocyte-derived sclerostin inhibits bone formation: its role in bone morphogenetic protein and Wnt signaling. J Bone Joint Surg Am 90 Suppl 1:31–35
21. Torreggiani E, Matthews BG, Pejda S, Matic I, Horowitz MC, Grcevic D, Kalajzic I (2013) Preosteocytes/osteocytes have the potential to dedifferentiate becoming a source of osteoblasts. PLoS One 8:e75204

22. van Bezooijen RL, Svensson JP, Eefting D, Visser A, van der Horst G, Karperien M et al (2007) Wnt but not BMP signaling is involved in the inhibitory action of sclerostin on BMP-stimulated bone formation. J Bone Miner Res 22:19–28
23. Xiong J, Onal M, Jilka RL, Weinstein RS, Manolagas SC, O'Brien CA (2011) Matrix-embedded cells control osteoclast formation. Nat Med 17:1235–1241
24. Yang W, Harris MA, Heinrich JG, Guo D, Bonewald LF, Harris SE (2009) Gene expression signatures of a fibroblastoid preosteoblast and cuboidal osteoblast cell model compared to the MLO-Y4 osteocyte cell model. Bone 44:32–45