Full title

Comparative gene expression analysis for pre-osteoblast MC3T3-E1 cells under non-adhesive culture toward osteocyte differentiation

Short title

Non-adhesive culture toward osteocyte differentiation

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Abstract

Osteocytes play an important role to modulate the bone remodeling and are also known as terminally differentiated cells originated from the osteoblast precursor cells, but its differentiation mechanism remains unclear. Since an efficient *in vitro* method to evoke the osteocyte differentiation from the osteoblast precursor cells has not been established, we conducted the comparative gene expression analysis for mouse pre-osteoblast MC3T3-E1 cells in order to elucidate the key factors to induce the osteocyte differentiation from the pre-osteoblast cells. In this study, we prepared four different culture environments by modulating their cell-substrate interaction and cell-cell interaction; 1) low and 2) high cell density on the adhesive culture models, and 3) low and 4) high cell density on the non-adhesive floating culture models. By comparing these conditions in terms of cell-substrate and cell-cell interaction, we showed that the elimination of cell-substrate interaction under non-adhesive floating culture greatly up-regulated the gene expression of osteocyte markers in the pre-osteoblast cells. Moreover, the presence of moderate cell-cell interaction in the non-adhesive spheroid culture further enhanced the up-regulation of osteocyte markers for the pre-osteoblast cells. The results altogether suggest the most appropriate culture environment to induce the *in vitro* osteocyte differentiation of pre-osteoblast cells.

Introduction

Osteocytes are mechanosensory cells playing an important role as a control tower in the bone to modulate the bone remodeling by communicating with osteoblasts and osteoclasts (1–3). While the osteoblasts secret the organic matrix to undergo the bone formation, their role has been studied using a two-dimensional (2D) cell-adhesive rigid plastic culture dish for several weeks (4–7), so that these *in vitro* studies broadened our knowledge understanding the properties and role of the osteoblasts *in vivo*. On the other hand, while the osteocytes comprise

of more than 90% of bone cells (8), there are difficulties in isolation and cultivation methods. Since the osteocytes are deeply buried inside the rigid mineralized bone matrix, the isolation of the primary osteocytes is relatively difficult to other cell sources. Although the isolation methods for the primary osteocytes have been recently introduced by several groups (9–12), a long-term culture is even more challenging on the normal cell-adhesive culture dish. Due to those difficulties, the long period culture of osteoblasts has been often utilized for the osteocyte study as surrogate (13), because the osteocyte is known as terminally differentiated cells from the osteoblast precursor cells (14). However, with regard to cell population of osteocytes obtained from the osteoblast culture and the long period of culture time, the efficient method to induce the osteocyte differentiation from the osteoblast precursor cells has not been established yet.

Previously, our group reported that a three-dimensional (3D) culture method triggers the osteocyte differentiation of osteoblast precursor cells in a short period of culture time, within 2 days, compared to the conventional 2D monolayer condition (15–17). Although we figured out the cell condensed condition acquired from the 3D culture model elicited the transient up-regulation of osteocyte markers, the detailed mechanism still remained unknown. In this study, we attempted to find out the key factors to induce the osteocyte differentiation of pre-osteoblast cells by comparison studies using several culture models. Particularly, we focused on the involvement of cell-substrate interaction and cell-cell interaction against the osteocyte differentiation.

Materials and Methods

Cell culture

In this study, we utilized the mouse pre-osteoblast cell line, MC3T3-E1, which was provided by RIKEN BRC (Tsukuba, Japan). The cells were cultured in α-MEM (Gibco, New York, USA) supplemented with 10% fetal bovine serum (Gibco, New York, USA) and 1% antibiotic-antimycotic solution (Gibco, New York, USA) in a humidified incubator at 37 °C with 5% CO_2. Cell passage was carried out every 2 – 3 days when the cell confluency became about 80%.

Preparation of four different culture models

To prepare for four different culture models, we subcultured MC3T3-E1 cells in the basal culture medium in the absence of osteogenic supplements such as ascorbic acid or β-glycerophosphate. For adhesive culture models, the cells were subcultured in 35 mm cell-adhesive petri dishes (ThermoFisher Scientific, Massachusetts, USA). To prepare for sparse and dense cell confluency, we subcultured the cells for 2 days on the petri dishes at two different cell densities as shown in Fig. 1A – C; low cell density (52 cells/mm^2; 2D LD) and high cell density (208 cells/mm^2; 2D HD), respectively. For non-adhesive floating culture model, we introduced two different conditions termed floating singular cells with low cell density (FL LD) and floating spheroid with high cell density (FL HD) as described in Fig. 1D – F. The FL LD were prepared by subculturing sparse cells for 2 days in a 6-well ultra-low attachment flat-bottom dish (Corning Coaster, New York, USA) (16,667 cells/ml). For the FL HD model, we utilized a Nunclon Sphera 96-well ultra-low attachment round-bottom dish (ThermoFisher Scientific, Massachusetts, USA) as described in the previous study (16). Due to ease of use for further assay using real-time PCR, 2,500 cells (25,000 cells/ml) were subcultured for 2 days to fabricate the FL HD.

Real-time PCR

The samples were collected in 1 ml of Isogen II (Nippon Gene, Toyama, Japan) and stored at –80 ℃ until the RNA extraction. For the RNA extraction, 50 μl of p-Bromoanisole (Nacalai Tesque, Kyoto, Japan) was added to the sample in Isogen II. After mixing the contents of the tube, the tube was centrifuged at 12,000 g for 10 minutes at 4 ℃. The supernatants were then isolated from the tube and transferred to the spin cartridge (PureLink RNA Mini Kit, Invitrogen, Massachusetts, USA). According to the manufacturer's protocol, the RNA was extracted from the sample. Then, cDNA was synthesized from the RNA using the Transcriptor Universal cDNA Master (Roche, Mannheim, Germany). To perform real-time PCR, PowerUp SYBR Green Master mix (ThermoFisher Scientific, Massachusetts, USA) was utilized. To measure the differentiation marker of osteocytes, we examined the mRNA expression of osteopontin (*Opn*), the dentin matrix protein 1 (*Dmp1*), and sclerostin (*Sost*). As a reference gene, we used glyceraldehyde-phosphate dehydrogenase (*Gapdh*) to normalize all the target mRNA expressions. The sets of gene-specific oligonucleotide primers are described in Table 1. We analyzed changes in a target sample relative to a control sample to show the fold change of mRNA expression in the sample.

Statistical analysis

A Student's *t*-test or one-way analysis of variance with Tukey's honestly significant different (HSD) *post hoc* test (with $\alpha = 0.05$) was assessed to evaluate the statistical significance. We assume that *p*-value of 0.05 or less were considered to be significant.

Results

Non-adhesive culture at low and high cell density highly up-regulated gene expressions of osteocyte markers in pre-osteoblast cells.

As illustrated in Fig. 2A, we introduced two types of low cell density culture model in order to examine the effect of cell-substrate interaction with the least effect of cell-cell interaction on the osteocyte differentiation. While the conventional 2D model at low cell density model was prepared by subculturing MC3T3-E1 cells at 52 cells/mm^2, which had the sparse subcultured cells on the adhesive culture dish after 2 days in order to provide the low cell-cell interaction, termed 2D LD. On the other hand, MC3T3-E1 cells at low cell density (16,667 cells/ml) was subcultured on the non-adhesive ultra-low attachment culture dish. After 2 days, floating singular cells were formed without attaching to the neighboring cells in the absence of cell-substrate interaction on the culture dish. We defined these floating singular cells as FL LD. After 2-day culture, we collected those two samples and then measured expression changes in osteocyte gene markers measured by real-time PCR analysis in Fig. 2B. As a result, all the mRNA expressions for osteocyte markers in FL LD were remarkably up-regulated compared to the 2D LD; *Opn* (120-fold change; $p < 0.005$), *Dmp1* (4,550-fold change; $p < 0.005$), and *Sost* (39.6-fold change; $p < 0.05$). Within a short time, 2 days, the up-regulations of osteocyte mRNA expressions were acquired from the non-adhesive culture at the low cell density.

In order to elucidate the effect of cell-substrate interaction on osteocyte differentiation in the presence of cell-cell interaction, we prepared two other culture models. As described in Fig. 2C, the 2D monolayer model was fabricated by subculturing MC3T3-E1 cells at high cell density (208 cells/mm^2) to become fully confluent after 2 days of culture. This monolayer model exhibited the cell-substrate interaction as well as the cell-cell interaction. We termed this as 2D HD. As a comparative model, we utilized a spheroid model subcultured on the non-adhesive plate while the cells in the spheroids were surrounded by the neighboring cells to provide the moderate cell-cell interaction with high cell density. We termed this spheroid model under the floating culture as FL HD. In Fig. 1F, the spheroid was fabricated by

146 subculturing 2,500 cells on the ultra-low attachment culture plate. After 2-day culture, a
147 diameter of the spheroid became about 125 μm. We evaluated gene expression changes
148 between these two models measured by real-time PCR in Fig. 2D. As a result, compared to the
149 2D HD model, all the mRNA expressions for osteocyte markers in FL HD were greatly up-
150 regulated; *Opn* (68.2-fold change; $p < 0.005$), *Dmp1* (1,120-fold change; $p < 0.005$), and *Sost*
151 (271.2-fold change; $p < 0.05$). Hence, the non-adhesive floating culture at high cell density
152 greatly up-regulated the mRNA expressions of osteocyte markers for pre-osteoblast cells
153 within 2 days.
154
155 **Moderate cell-cell interaction under the non-adhesive spheroid culture condition further**
156 **promoted the up-regulations of osteocyte markers in pre-osteoblast cells.**
157 To examine the effect of cell-cell interaction on the osteocyte differentiation under the adhesive
158 culture, we compared the gene expressions in two conventional adhesive models, 2D LD and
159 2D HD as illustrated in Fig. 3A. By altering the cell densities subcultured on the adhesive
160 culture plate (2D LD: 52 cells/mm^2; 2D HD: 208 cells/mm^2), we prepared the 2D sparse and
161 dense cellular culture model after 2 days of culture period, which provided less and greater
162 cell-cell interactions with the cell-substrate interaction on the culture dish, respectively. As a
163 result of real-time PCR assay in Fig. 3B, there was no significant change in osteocyte markers
164 between 2D LD and 2D HD; *Opn* (0.86-fold change; $p = 0.57$), *Dmp1* (1.09-fold change; $p =$
165 0.91), and *Sost* (1.19-fold change; $p = 0.76$).

166 In order to examine the involvement of cell-cell interaction on the osteocyte differentiation
167 under the non-adhesive floating culture, we compared the gene expression levels of osteocyte
168 markers in FL LD and FL HD. While the FL LD is a singular cell model with the minimized
169 cell-cell interaction as described in the Fig. 3C, the FL HD consists of 2,500 cells surrounded
170 by neighboring cells which has the high cell density with the greater cell-cell interaction per

cells. Real-time PCR results in Fig. 3D represented that *Opn* (0.61-fold change; $p < 0.05$) and *Dmp1* (0.13-fold change; $p < 0.005$) were significantly down-regulated in those in FL HD, compared to those in FL LD. On the other hand, the mature osteocyte marker, *Sost,* were remarkably up-regulated in the FL HD compared to the FL LD (46.5-fold change; $p < 0.05$).

Osteocyte markers in cells dissociated from the spheroids on the adhesive culture were greatly down-regulated.

In order to clarify the effect of cell-substrate interaction on the osteocyte differentiation, we introduced another model termed spheroid-to-monolayer model (FL-2D HD). After the FL HD was fabricated for 2 days in the non-adhesive ultra-low attachment culture dish, the 2-day old FL HD was transferred to a normal adhesive culture dish as illustrated in Fig. 4A. Immediate after transferring, the FL HD attached to the adhesive culture dish. In Fig. 4B, the cells from the FL HD initiated to spread over the culture dish at 6-hour after transferring. Eventually, the FL HD was fully dissociated and became like 2D HD model after 48 hours as shown in Fig. 4C. The cells dissociated from the FL HD was termed FL-2D HD. To evaluate the gene expression changes in the FL-2D HD, we compared it to those in 2D HD and FL HD after 4-day incubation. The real-time PCR result in Fig. 4D represented that all the osteocyte markers in the FL HD were highly up-regulated compared to those in the 2D HD, whereas the significant down-regulations of osteocyte markers were observed in the FL-2D HD model compared to the FL HD.

Discussion

Our group reported that the 3D culture system triggers the osteocyte differentiation of osteoblast precursor cells (15–18). While the 3D culture models of osteoblast precursor cells

exhibited the osteocyte-likeness within a short time, the triggering factors for osteocyte-likeness acquired in the 3D culture model still remained unclear. In this study, we attempted to elucidate the key factors to induce the osteocyte differentiation of pre-osteoblast cells by comparison studies of gene expressions using several culture models. Particularly, we focused on the effects of cell-substrate interaction and cell-cell interaction on the osteocyte differentiation markers by altering the cultural environments for pre-osteoblast cells.

In Fig. 2, we represented that the non-adhesive floating culture for pre-osteoblast cells exhibited the greater gene expression of osteocyte markers within 2 days beyond the adhesive culture methods, regardless of its cell density. The results implied that the elimination of cell-substrate interaction for pre-osteoblasts induced the transient up-regulations of osteocyte markers. For the adhesive culture model in Fig. 3A – B, the gene expressions of osteocyte markers were not altered depending on the cell density. Corresponding to the conventional 2D model on the adhesive culture, it was difficult to achieve such a dramatic change in gene expression of the osteocyte markers in a short period of culture time. On the other hand, in the non-adhesive floating culture represented in Fig. 3C – D, the FL HD model rendered even greater mRNA expressions of mature osteocyte marker, *Sost,* compared to that of FL LD. Regarding the significant reductions of *Opn* and *Dmp1* mRNA expressions in the FL HD model, *Opn* and *Dmp1* expressions were highly detected in relatively immature osteocytes (1,14,19,20), so that the FL LD rendered the immature osteocyte-likeness compared to the FL HD model. On the other hand, since the FL HD showed a greater gene expression of mature osteocyte marker, *Sost,* it implies the FL HD exhibited the mature osteocyte-likeness beyond the FL LD.

FL HD model exhibited the matured osteocyte-likeness. As Fujita's group reported that the direct cell-cell contact enhanced the differentiation of osteocytes (21), the present study implies

219 that the cell-cell interaction further promoted the up-regulation of osteocyte markers under the
220 floating culture. Inside the FL HD model, the cells are surrounded by neighboring cells and
221 encountered the greater cell-cell interaction compared to other models such as 2D LD, 2D HD,
222 and FL LD. The enhancement of the cell-cell interaction contributed to the up-regulation of
223 osteocyte markers. By transferring the FL HD to the normal adhesive culture dish in Fig. 4, the
224 FL HD attained the cell-substrate interaction and became like 2D HD model after 2 days.
225 Consequently, the dissociated FL-2D HD model deprived the up-related gene expression of
226 osteocyte markers acquired from the FL HD. Hence, the floating culture removing the cell-
227 substrate interaction becomes a useful experimental method for the osteocyte differentiation
228 studies while the cell-cell interaction further enhances the osteocyte differentiation under the
229 floating spheroid culture, as described in Fig. 5. In other words, the 3D culture model such as
230 spheroid model has a potential to become an *in vitro* osteocyte differentiation model.

231 Our group previously showed that the cell-fate decision of mesenchymal stem cells toward the
232 osteocyte differentiation is committed by actin balancing in the 3D spheroid culture (18). With
233 regard to the involvement of actin balancing, the floating culture for pre-osteoblast cells is
234 thought to have less actin filament generation compared to the adhesive culture, so that the pre-
235 osteoblast cells under the floating culture exerted the osteocyte-likeness, whereas the
236 conventional model using the adhesive culture dish undergoes the osteoblast differentiation via
237 generation of actin filaments on the culture dish (22). Mullen's group also represented the
238 involvement of actin filaments in the osteocyte differentiation of MC3T3-E1 cell subcultured
239 on collagen matrices (23). Moreover, the gene expression of osteocyte markers was enhanced
240 by subculturing cells in a softer collagen substrate when compared to cells on a stiffer substrate
241 (24). Due to the stiffness changes, the alteration of actin filaments generated on the substrate
242 induced the gene expression changes of osteocyte markers. Hence, adhesive/non-adhesive
243 cultural environments in this study are thought to elicit the alteration in the actin balancing and

244 further to modulate the osteocyte differentiation.

245 From other studies related to the osteocyte differentiation on the 2D conventional adhesive
246 models, the pre-osteoblast cells are known to induce the osteoblast differentiation as well as
247 bone mineralization after several weeks to several months of culture. After the overly confluent
248 condition on the 2D adhesive culture, the subcultured cells form 3D dome-shape of bone-like
249 nodules in the culture dish and eventually rendered the bone mineralization accompanied by
250 osteocyte differentiation (25). Moreover, the osteocyte-like cells were also observed in the
251 bone-like nodules (26). Inside this bone nodule, the cells were highly surrounded by
252 neighboring cells and did not have the culture environment attached on to the 2D adhesive dish
253 to generate the actin filament, so that the osteocyte differentiation is consequently occurred
254 inside the nodule. Previous studies by our group suggested that the cell condensation condition
255 acquired from the 3D spheroid model triggered osteocyte differentiation of pre-osteoblast cells
256 (15,16) and mesenchymal stem cells (18). Hence, both bone-like nodule and spheroid cultures
257 might provide those suitable culture environments not to generate actin filaments, eventually
258 undertaking the osteocyte differentiation. Our present study also supports this claim that the
259 elimination of cell-substrate interaction achieved in the floating culture reduced the generation
260 of actin filaments and consequently evoked the osteocyte differentiation of pre-osteoblast cells.

261 *In vivo*, the osteoblasts are placed on the hard bone matrix and secretes the organic matrix for
262 bone formation during the bone remodeling process (5). As many researchers conducted the
263 osteoblast differentiation study over the last several decades, the conventional 2D culture
264 model on the plastic or glass culture plate has successfully broadened our knowledge on the
265 cellular behaviors including proliferation and differentiation for the osteoblasts because the 2D
266 culture model mimicked the strong cell-substrate interaction of the osteoblasts as *in vivo*
267 situation placed on the hard bone matrix. Regarding the osteocyte study, however, the efficient

method to induce the osteocyte differentiation was yet to be established while the osteocyte differentiation was thought to be achieved beyond the osteoblast differentiation. *In vivo,* the osteocytes are embedded inside the hard bone matrix, but their cell body with processes are placed inside a small space called lacunae which is filled with interstitial fluid (27). In other words, the cell body of osteocyte was not directly attached on to the bone matrix, but with its cellular processes via pericellular glycocalyx and integrins (28,29). Our present study might imply that the current floating culture model mimics this *in vivo* situation with minimization of cell-substrate interaction with less actin formation, resulting in remarkable up-regulations of osteocyte markers within a short time. Despite such dramatic changes in the osteocyte gene expressions under the floating culture shown in this study, dendritic processes of osteocytes have not been observed in the current models so that further studies will be required to acquire a complete osteocyte differentiation with morphological features.

In conclusion, we elucidated the effect of culture environments on the osteocyte differentiation, particularly cell-substrate interaction and cell-cell interaction. We showed that the elimination of cell-substrate interaction in the floating culture greatly up-regulated the osteocyte markers of the pre-osteoblast cells within 2 days. Moreover, the moderate cell-cell interaction acquired from the spheroid under the floating culture further enhanced the up-regulation of osteocyte markers. From this study, we suggest a new strategy for *in vitro* osteocyte differentiation studies.

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References

1. **Bonewald, L.F.:** Mechanosensation and transduction in osteocytes, Bonekey Osteovision, **3**, 7–15 (2006).
2. **Adachi, T., Aonuma, Y., Ito, S., Tanaka, M., Hojo, M., Takano-Yamamoto, T., and Kamioka H.:** Osteocyte calcium signaling response to bone matrix deformation, J. Biomech., **42**, 2507–2512 (2009).
3. **Qin, L., Liu, W., Cao, H., and Xiao, G.:** Molecular mechanosensors in osteocytes, Bone Res., **8**, 1–24 (2020).
4. **Wang, D., Christensen, K., Chawla, K., Xiao, G., Krebsbach, P.H., and Franceschi, R.T.:** Isolation and characterization of MC3T3-E1 vivo differentiation / mineralization potential, J. Bone Miner. Res., **14**, 893–903 (1991).
5. **Blair, H.C., Larrouture, Q.C., Li, Y., Lin, H., Beer-Stoltz, D., Liu, L., Tuan, R.S., Robinson, L., Schlesinger, P.H., and Nelson, D.J.:** Osteoblast differentiation and bone matrix formation in vivo and in vitro, Tissue Eng. Part B Rev., **23**, 268–280 (2017).
6. **Bielby, R.C., Boccaccini, A.R., Polak, J.M., and Buttery, L.D.K.:** In vitro differentiation and in vivo mineralization of osteogenic cells derived from human embryonic stem cells, Tissue Eng., **10**, 9–10, (2004).
7. **Woo, S.M., Rosser, J., Dusevich, V., Kalajzic, I., and Bonewald, L.F.:** 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 (2011).

8. **Bonewald, L.F.:** The amazing osteocyte. J. Bone Miner. Res., **26**, 229–238 (2011).
9. **Mikuni-Takagaki, Y., Kakai, Y., Satoyoshi, M., Kawano, E., Suzuki, Y., Kawase, T., and Saito, S.:** Matrix mineralization and the differentiation of osteocyte-like cells in culture, J. Bone Miner. Res., **10**, 231–242 (1995).
10. **Tanaka-Kamioka, K., Kamioka, H., Ris, H., and Lim, S.S.:** Osteocyte shape is dependent on actin filaments and osteocyte processes are unique actin-rich projections, J. Bone Miner. Res., **13**, 1555–1568 (1998).
11. **Prideaux, M., Schutz, C., Wijenayaka, A.R., Findlay, D.M., Campbell, D.G., Solomon, L.B., and Atkins, G.:** Isolation of osteocytes from human trabecular bone, Bone., **88**, 64–72 (2016).
12. **Shah, K.M., Stern, M.M., Stern, A.R., Pathak, J.L., Bravenboer, N., and Bakker, A.D.:** Osteocyte isolation and culture methods, Bonekey Rep., **5**, 838 (2016).
13. **Bakker, A.D. and Klein-Nulend, J.:** Role of osteocytes in the adaptation of bone to mechanical loading, Fut. Rheumatol., **3**, 571–586 (2008).
14. **Dallas, S.L., Prideaux, M., and Bonewald, L.F.:** The osteocyte: An endocrine cell . . . and more, Endocr. Rev., **34**, 658–690 (2013).
15. **Kim, J. and Adachi, T.:** Cell condensation triggers the differentiation of osteoblast precursor cells to osteocyte-like cells, Front. Bioeng. Biotechnol., **7**, 288 (2019).
16. **Kim, J., Kigami, H., and Adachi, T.:** Characterization of self-organized osteocytic spheroids using mouse osteoblast-like cells, J. Biomech. Sci. Eng., **15**, 20-00227 (2020).
17. **Kim, J. and Adachi, T.:** Modulation of Sost gene expression under hypoxia in 3D scaffold-free osteocytic tissue, Tissue Eng. Part A., **27**, 1037-1043 (2021).

18. **Kim, J. and Adachi, T.:** Cell-fate decision of mesenchymal stem cells toward osteocyte differentiation is committed by spheroid culture, Sci. Rep., **11**, 13204 (2021).
19. **Robling, A.G. and Bonewald, L.F.:** The Osteocyte : New Insights, Annu. Rev. Physiol., **82**, 485–506 (2020).
20. **Kim, J., Ishikawa, K., Sunaga, J., and Adachi, T.:** Uniaxially fixed mechanical boundary condition elicits cellular alignment in collagen matrix with induction of osteogenesis, Sci. Rep., **11**, 9009 (2021).
21. **Fujita, K., Xing, Q., Khosla, S., and Monroe, D.G.:** Mutual enhancement of differentiation of osteoblasts and osteocytes occurs through direct cell-cell contact, J. Cell. Biochem., **115**, 11 (2014).
22. **Chen, L., Shi, K., Frary, C.E., Ditzel, N., Hu, H., Qiu, W., and Kassem, M.:** Inhibiting actin depolymerization enhances osteoblast differentiation and bone formation in human stromal stem cells, Stem Cell Res., **15**, 281–289 (2015).
23. **Mullen, C.A., Haugh, M.G., Schaffler, M.B., Majeska, R.J., and McNamara, L.M.:** Osteocyte differentiation is regulated by extracellular matrix stiffness and intercellular separation, J. Mech. Behav. Biomed., **28**, 183-194 (2013).
24. **McGarrigle, M.J., Mullen, C.A., Haugh, M.G., Voisin, M.C., and McNamara, L.M.:** Osteocyte differentiation and the formation of an interconnected cellular network in vitro, Eur. Cell. Mater., **24**, 323-340 (2016).
25. **Bhargava, U., Bar-Lev, M., Bellows, C.G., and Aubin, J.E.:** Ultrastructural analysis of bone nodules formed in vitro by isolated fetal rat calvaria cells, Bone, **9**, 155–163 (1988).
26. **Kawai, S., Yoshitomi, H., Sunaga, J., Alev, C., Nagata, S., Nishio, M., Hada, M., Koyama, Y., Uemura, M., Sekiguchi, K., and other 9 authors:** In vitro bone-like

nodules generated from patient-derived iPSCs recapitulate pathological bone phenotypes, Nat. Biomed. Eng., **3**, 558-570 (2019).

27. **Lai, X., Price, C., Modla, S., Thompson, W.R., Caplan, J., Kirn-Safran, C.B., and Wang, L.:** The dependences of osteocyte network on bone compartment, age, and disease, Bone Res., **3**, 15009 (2015).

28. **Wang, B., Lai, X., Price, C., Thompson, W.R., Li, W., Quabili, T.R., Tseng, W.J., Liu, X.S., Zhang, H., Pan, J., and other 3 authors:** Perlecan-containing pericellular matrix regulates solute transport and mechanosensing within the osteocyte lacunar-canalicular system, J. Bone Miner. Res., **29**, 878–891 (2014).

29. **Cabahug-Zuckerman, P., Stout, R.F., Majeska, R.J., Thi, M.M., Spray, D.C., Weinbaum, S., and Schaffler, M.B.:** Potential role for a specialized β 3 integrin-based structure on osteocyte processes in bone mechanosensation, J. Orthop. Res., **36**, 642–652 (2018).

Figure legends

Figure 1. Four culture models using MC3T3-E1 at low and high cell density in adhesive and non-adhesive culture condition. (A) Schematic illustration of adhesive culture model for MC3T3-E1 at low cell density (2D LD) and high cell density (2D HD). Microscopic images of adhesive culture model for MC3T3-E1 (B) at low cell density (2D LD) and (C) at high cell density (2D HD). Scale bars represent 200 μm. (D) Schematic illustration of non-adhesive culture model for MC3T3-E1 at low cell density (FL LD) and high cell density (FL HD). Microscopic images of adhesive culture model for MC3T3-E1 (E) at low cell density (FL LD) and (F) at high cell density (FL HD). Scale bars represent 200 μm.

Figure 2. Effect of culture condition on osteocyte gene expressions of MC3T3-E1 at low and

high cell density. (A) Schematic illustration of low cell density for MC3T3-E1 in adhesive culture (2D LD) and non-adhesive culture (FL LD). (B) Relative mRNA expressions of genes in MC3T3-E1 under 2D LD and FL LD culture after 2 d incubation. The mRNA expression of *Opn*, *Dmp1*, and *Sost* was measured by real-time PCR and normalized to that of *Gapdh*. Bars represent the mean ± standard error (n = 4; p-value was calculated by Student's t-test; * $p < 0.05$, ** $p < 0.005$). (C) Schematic illustration of high cell density for MC3T3-E1 in adhesive culture (2D HD) and non-adhesive culture (FL HD). (D) Relative mRNA expressions of genes in MC3T3-E1 under 2D HD and FL HD culture after 2 d incubation. The mRNA expression of *Opn*, *Dmp1*, and *Sost* was measured by real-time PCR and normalized to that of *Gapdh*. Each value was then normalized to the control sample. Bars represent the mean ± standard error (n = 10; p-value was calculated by Student's t-test; * $p < 0.05$, ** $p < 0.005$).

Figure 3. Effect of cell density on osteocyte gene expressions of MC3T3-E1 under adhesive and non-adhesive culture condition. (A) Schematic illustration of adhesive culture model for MC3T3-E1 at low cell density (2D LD) and high cell density (2D HD). (B) Relative mRNA expressions of genes in MC3T3-E1 under 2D LD and 2D HD culture after 2 d incubation. The mRNA expression of *Opn*, *Dmp1*, and *Sost* was measured by real-time PCR and normalized to that of *Gapdh*. Bars represent the mean ± standard error (n = 4; p-value was calculated by Student's t-test; * $p < 0.05$, ** $p < 0.005$). (C) Schematic illustration of non-adhesive culture model for MC3T3-E1 at low cell density (FL LD) and high cell density (FL HD). (D) Relative mRNA expressions of genes in MC3T3-E1 under FL LD and FL HD culture after 2 d incubation. The mRNA expression of *Opn*, *Dmp1*, and *Sost* was measured by real-time PCR and normalized to that of *Gapdh*. Each value was then normalized to the control sample. Bars represent

the mean ± standard error ($n = 6$; p-value was calculated by Student's t-test; * $p < 0.05$, ** $p < 0.005$).

Figure 4. Osteocyte gene expression change in cells dissociated from the spheroids. **(A)** Schematic illustration of MC3T3-E1 cells dissociated from the spheroids (FL-2D HD) by transferring the spheroid subcultured for 2 d in the non-adhesive floating culture dish into the adhesive culture dish and subcultured them for extra 2 d. Microscopic images of dissociated cells from spheroids at (B) 6 h and (C) 48 h after transferring. Dotted arrows indicate the spheroid. Scale bars indicate 200 μm. (D) Relative mRNA expression of *Opn*, *Dmp1*, and *Sost* in MC3T3-E1 under 4-day-old 2D HD, 4-day-old FL HD, and FL-2D HD culture after 2 d incubation was measured by real-time PCR and normalized to that of *Gapdh*. Each value was then normalized to the control sample. Bars represent the mean ± standard error ($n = 6$; p-value was calculated by ANOVA with Tukey's HSD *post-hoc* test ($\alpha = 0.05$); Bar indicates the significance between groups).

Figure 5. Schematic diagram illustrating the summary of comparative osteocyte gene expression analysis for pre-osteoblast MC3T3-E1 cells at low and high cell density under adhesive and non-adhesive culture dish.