Title

Modulation of *Sost* gene expression under hypoxia in 3D scaffold-free osteocytic tissue

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Abstract

Bone-related studies have been widely carried out by culturing cells on two-dimensional (2D) culture system due to its easiness of handling, but these 2D *in vitro* achievements may imply a distinct outcome compared to the *in vivo* situation. On the other hand, three-dimensional (3D) culture system has been suggested as a better biomimetic *in vitro* model by providing an appropriate cell-cell or cell-matrix interaction. In this study, we successfully reconstructed a 3D disk type of scaffold-free tissue (SFT) using mouse osteoblast-like cells, which evoked an osteocyte differentiation within 2 days. Particularly, the SFT was also utilized as an *in vitro* osteocytic model to elucidate the effect of hypoxia on cellular differentiation capability. As a result, the hypoxia up-regulated a matured osteocyte marker, *Sost*, in the SFT, whereas both osteoblast and osteocyte markers were significantly down-regulated by hypoxia in the 2D conventional monolayer model. The results imply that the hypoxia may enhance the initiation of osteocyte differentiation and retain the osteocyte differentiation in the 3D culture system. Notably, we reported the significance of 3D culture system which might represent the *in vivo* situation regarding cellular response to stimuli. Hence, our study suggests wide applications of SFT using osteoblast cells as a novel *in vitro* osteocyte model for the osteocyte-related studies.

Impact statement

In this study, we fabricated a 3D disk type of scaffold-free osteocytic tissue, termed scaffold-free tissue (SFT), reconstructed by mouse osteoblast-like cells. It induced an osteocyte differentiation of osteoblast-like cells in the SFT within 2 days. Moreover, we first showed that a matured osteocyte marker, *Sost*, was modulated by hypoxia in the SFT in a different manner

compared to the 2D monolayer. These results highlighted the significance of 3D culture system which might represent the *in vivo* situation regarding cellular response to stimuli. Notably, our model can be utilized as a new *in vitro* osteocyte model for the osteocyte-related studies.

Introduction

Osteocytes refer to as bone cells embedded inside the mineralized bone matrix, comprising more than 90% of the bone tissue. The osteocytes are responsible for sensing mechanical stimuli and transducing biochemical signals to osteoclasts for bone resorption and osteoblasts for bone formation[1,2]. While the mechanical stimuli have been considered as an important factor for cellular behaviors and functions[3–6], relatively few studies are available for the osteocytes since they are embedded inside the hard mineralized bone matrix, causing a difficulty in accessibility to isolate the primary cells. Although the method of isolation and cultivation of osteocytes has been recently reported[7], it is still challenging to maintain the osteocyte-likeness *in vitro*. As a surrogate of osteocyte, osteoblastic cells have been widely utilized in many stuides[8].

While the osteocytes are captured in the mineralized bone matrix, they are subjected to hypoxic condition in a narrow space because they reside distant from the blood vessels[9,10]. Due to practical reasons such as a difficulty in accessibility to the osteocytes *in vivo*, a physiological level of oxygen concentration applied to the osteocytes has not been reported. However, several studies have attempted to apply this hypoxic condition in order to figure out its effect on the osteoblasts/osteocytes. The hypoxic condition using 1 or 5% O_2 concentration modulated the transformation to osteocytes from osteoblasts[11] or *ORP150* expression levels in mouse

64 osteoblast-like MC3T3-E1 cells[12]. As reported by those groups, we assumed that the hypoxia
65 plays a significant role in cellular differentiation capability for osteocytes.

66 Recently, application of three-dimensional (3D) scaffold-free tissue engineering techniques,
67 referred to as organoids, have been widely broadened in the field of fundamental biology,
68 particularly in human development, disease, or drug screening. Compared to a conventional
69 two-dimensional (2D) culture technique, the 3D culture system is regarded as a more
70 biomimetic tissue models by providing an appropriate cell-cell or cell-matrix interaction[13].
71 While most of the fundamental biology on cellular behaviors or functions of osteocytes were
72 studied using the 2D culture system, we attempted to fabricate a novel 3D tissue engineered
73 osteocytic model and further to elucidate its response to hypoxia.

74

75 **Materials and method**

76 **Cell culture and hypoxia**

77 Mouse osteoblast-like cells, MC3T3-E1, were provided by RIKEN Cell Bank and cultured in
78 α-MEM (Gibco) containing 10% FBS (Gibco) and 1% antibiotic-antimyotic (Gibco) in a
79 humidified incubator at 37 °C with 5% CO_2. When the cells become 80 – 90 % confluent, a
80 cell passage was carried out. In this study, we applied a hypoxic condition to samples using a
81 BIONIX hypoxic culture kit (Sugiyama-gen). According to the manufacturer's manual, the
82 samples were subjected to hypoxia (5% O_2) for 48 hours during the fabrication process of SFT
83 (Day 0 – Day 2) or 2 days after the SFT was fabricated (Day 2 – Day 4).

84

85

86 **Fabrication of scaffold-free tissue**

87 In order to prepare for a scaffold-free tissue (SFT), the MC3T3-E1 cells were subcultured at a
88 cell density of 50,000 cells (1,300 cells/mm^2) in a 7 mm diameter of glass ring which was set
89 on the culture dish as illustrated in Fig. 1(A). A monolayer of the cells was formed inside the
90 ring by culturing for 2 days. Then, 2,000,000 cells (51,995 cells/mm^2) were subcultured on top
91 of the monolayer inside the ring. The monolayer embedded helped to retain the disk shape
92 inside the ring as described in the Fig. 1 (B). After 3 hours of incubation, the ring was gently
93 removed to provide sufficient nutrients to a 3D tissue-engineered construct, termed SFT. As
94 represented in Fig. 1(C) and (D), the SFT was further incubated for 2 days according to
95 experiments. For control sample, 200,000 cells (208 cells/mm^2) were subcultured on a culture
96 dish to form a conventional 2D monolayer model reconstructed by MC3T3-E1 and incubated
97 to become fully confluent after 2 days of culture period in Fig. 1(E).

98

99 **Real-time PCR**

100 For RNA extraction, the samples were first washed once with PBS and collected with 1 ml of
101 Isogen II (Nippon Gene). After adding 50 μl of p-Bromoanisole (Nacalia Tesque), the tube was
102 vortexed thoroughly for 10 seconds and centrifuged at 12,000 g for 10 minutes at 4 °C.
103 Following centrifugation, the mixture was separated into two phases. A colorless upper
104 aqueous phase of the mixture was carefully transferred into a new microtube and mixed with
105 the same amount of 70% ethanol. After being vortexed, the mixture was transferred into the
106 spin cartridge (PureLink RNA Mini Kit; Invitrogen). According to the manufacturer's protocol,
107 the samples were then centrifuged, followed by washing process. The samples were then dried
108 and quickly added an appropriate amount of RNase free water. For cDNA synthesis and real-

time quantitative PCR, we utilized the Transcriptor Universal cDNA Master (Roche) and PowerUp SYBR Green Master Mix (ThermoFisher), respectively. We then investigated runt related transcription factor 2 (*Runx2*), alkaline phosphatase (*Alp*), and collagen, type I, alpha 1 chain (*Col1a1*) as osteoblast markers while osteopontin (*Opn*), the phosphate regulating endopeptidase homolog X-linked (*Phex*), the dentin matrix protein 1 (*Dmp1*), and sclerostin (*Sost*) were utilized as osteocyte markers. As hypoxia-regulated markers, we also examined hypoxia-inducible factor (*Hif1α*) and erythropoietin (*Epo*). All the PCR primers were described in Table 1 and classified as previous studies[14,15]. All the gene expressions were normalized to those of a reference gene, *Gapdh*.

Immunostaining

The SFT samples were washed twice with PBS and collected in the 4% paraformaldehyde (PFA) at room temperature for 15 minutes. After flushing twice with PBS, the samples were permeabilized using 0.1% triton X-100 at room temperature for 30 minutes. The samples were then washed twice with PBS and treated with 4% bovine serum albumin (Sigma) in PBS at room temperature for 1 hour to block non-specific interactions. After washing the blocking reagent, it is treated with the primary antibody, anti-DMP1 antibody (Abcam), overnight at 4 °C. Then, the samples were washed twice with PBS and incubated with the Alexa Flour 546 secondary antibody (Invitrogen), Alexa Fluor 488 Phalloidin (Invitrogen), and DAPI (Sigma) at room temperature for 1 hour. The stained samples were finally washed twice with PBS and preserved in PBS until the time of observation. The samples were observed using the FLUOVIEW FV3000 (Olympus).

Statistical analysis

The bars in the real-time PCR represent the mean ± standard error values. A Student's t-test or two-way ANOVA with Fisher's least significant difference (LSD) post hoc test (with $\alpha = 0.05$) was carried out to evaluate the statistical difference. *P*-value of 0.05 or less was assumed to be significant**.**

Results

Osteoblast-like cells in three-dimensional scaffold-free tissue enhanced osteocyte differentiation within 2 days

The SFT and monolayer reconstructed by osteoblast-like cells were incubated for 2 days. Real-time PCR analysis in Fig. 2 (A) showed that the osteoblast-like cells in the SFT had significant reductions in *Runx2* (0.30-fold change; $p < 0.005$), *Alp* (0.28-fold change; $p < 0.05$), and *Col1a1*(0.12-fold change; $p < 0.005$), compared to that in the monolayer. On the other hand, the SFT rendered remarkable up-regulations in the osteocyte mRNA expressions, such as *Opn* (1.79-fold change; $p < 0.05$), *Phex* (4.00-fold change; $p < 0.05$), *Dmp1* (23.3-fold change; $p < 0.05$), and *Sost* (17.8-fold change; $p < 0.05$). To elucidate the spatial information on osteocyte protein expression in the SFT, we conducted DMP1 immunostaining. DMP1 expression was examined because *Dmp1* mRNA expression in the SFT measured by real-time PCR was most up-regulated among the osteocyte markers. As described in Fig. 2 (B) – (E), the staining results showed that the DMP1 expression (green) was entirely detected in the SFT. The staining images also represented that the SFT had a thickness of about 50 μm.

Hypoxia promoted *Sost* gene expression for osteoblast-like cells in the scaffold-free tissue

In this experiment, we applied 5% hypoxic condition to monolayer and SFT for 48 hours during fabrication period (Day 0 – Day 2). Then, we evaluated changes in mRNA expressions of osteoblast and osteocyte markers in the absence or presence of hypoxia as shown in Fig. 3. By applying hypoxia, it reduced *Runx2* (0.67-fold change) and *Col1a1* (0.74-fold change) in the monolayer while the hypoxia suppressed *Runx2* (0.36-fold change), *Alp* (0.40-fold change), and *Col1a1* (0.41-fold change) mRNA expressions in the SFT. Applying hypoxia in the monolayer did not also alter the osteocyte mRNA expressions for 2 days; *Phex* (0.89-fold change), *Dmp1* (0.92-fold change), and *Sost* (1.15-fold change). On the other hand, the hypoxia significantly up-regulated *Sost* (2.46-fold change) mRNA expression in the SFT while *Phex* (0.96-fold change) and *Dmp1* (0.78-fold change) mRNA expressions were not modulated in the SFT. Furthermore, we also investigated *Hif1α* and *Epo* mRNA expression as hypoxia-related markers. The hypoxia significantly altered *Hif1α* (0.60-fold change) and *Epo* mRNA expressions (2.38-fold change) in the monolayer while it also greatly modulated *Hif1α* (0.36-fold change) and *Epo* mRNA expressions (6.74-fold change) in the SFT.

Hypoxia maintained up-regulation of *Sost* gene expression for osteoblast-like cells in the scaffold-free tissue

To investigate the effect of hypoxia after fabrication of SFT for maintenance of osteocyte differentiation, we conducted a hypoxic experiment between Day 2 and Day 4. In Fig. 4, applying hypoxia to the monolayer reconstructed by osteoblast-like cells significantly down-regulated all the mRNA expressions of osteoblast markers such as *Runx2* (0.39-fold change), *Alp* (0.52-fold change), and *Col1a1* (0.40-fold change), while the hypoxia also reduced the

177 *Runx2* (0.34-fold change), *Alp* (0.18-fold change), and *Col1a1* (0.30-fold change) mRNA
178 expressions in the SFT. On the other hand, in the monolayer, the hypoxia non-significantly
179 down-regulated the osteocyte gene expression; *Phex* (0.44-fold change), *Dmp1* (0.43-fold
180 change), and *Sost* (0.28-fold change). Regarding the SFT, the hypoxia did not alter the
181 osteocyte mRNA expression; *Phex* (0.88-fold change), *Dmp1* (0.89-fold change), and *Sost*
182 (1.79-fold change). Those results indicate that applying the hypoxia maintained the osteocyte
183 markers in the SFT. Moreover, we also examined the hypoxia-induced genes. The hypoxia
184 modulated *Hif1α* (0.46-fold change) and *Epo* mRNA expressions (0.56-fold change) in the
185 monolayer, whereas it altered *Hif1α* (0.44-fold change) and *Epo* mRNA expressions (1.25-fold
186 change) in the SFT.

187

188 **Discussion**

189 While three-dimensional (3D) *in vitro* model such as organoids have become a powerful tool
190 in the fundamental biological research, our group showed that osteoblast cells in the form of
191 3D spheroid structure triggered the osteocyte differentiation[16,17]. In this study, we developed a
192 novel disk type of 3D *in vitro* model, referred to as scaffold-free tissue (SFT), and further
193 investigated the capability of osteocyte differentiation for osteoblast-like cells in the form of
194 SFT compared to the 2D monolayer condition. In Fig. 2 (A), the osteocyte markers (*Opn*, *Phex*,
195 *Dmp1*, and *Sost*) were highly up-regulated in the SFT after 2 days of cultivation while it
196 suppressed the osteoblast mRNA expressions (*Runx2*, *Alp*, and *Col1*). Moreover, the
197 expression level of an osteocytic protein, DMP1, was detected entirely inside the SFT. Similar
198 to the previous studies[16,17], we showed that the osteoblast-like cells in the 3D culture system of

199 SFT exerted the osteocyte-likeness within 2 days, in the absence of chemical induction such as
200 osteogenic supplements.

201 In this study, we also examined the effect of hypoxia regarding changes in gene expression
202 levels of osteocyte differentiation markers. Since the osteocytes were embedded in the
203 mineralized bone, the osteocytes *in vivo* are thought to be subjected to the hypoxic condition.
204 Based on other reported study[11], we applied 5% hypoxia to the monolayer and SFT
205 reconstructed by osteoblast-like cells during fabrication of the SFT (Day 0 – 2). As a result of
206 applying hypoxic condition to the samples, the hypoxia-related genes, such as *Hif1α* and *Epo,*
207 were significantly altered as shown in Fig. 3. Our results regarding decrease in *Hif1α* and
208 increase in *Epo* genes under hypoxic condition correspond to other studies using the
209 conventional 2D model[18,19]. Particularly, we first represented that those hypoxia-related genes
210 were also modulated in the 3D SFT under hypoxia. In the monolayer condition, the hypoxia
211 suppressed osteoblast gene expressions within 2 days while there is no significant change in
212 osteocyte gene expressions as shown in Fig. 3. In the SFT, however, the hypoxia significantly
213 up-regulated *Sost* mRNA expression, whereas the *Sost* mRNA expression was not altered in
214 the monolayer. As the *Sost* gene is regarded as a matured osteocyte marker[14], the result may
215 indicate that the hypoxic condition in the 3D SFT culture system enhance the initiation of
216 osteocyte differentiation. Despite the up-regulation of *Sost* gene expression in the SFT under
217 hypoxia, further detailed study regarding the osteocyte differentiation need to be undertaken.

218 To examine the effect of hypoxia after the fabrication of SFT for maintenance of osteocyte
219 differentiation, we also applied the hypoxia on Day 2 for 48 hours (Day 2 – 4) and then
220 conducted real-time PCR in Fig 4. Similar to the result in Fig. 3, the hypoxia significantly
221 down-regulated all the osteoblast mRNA expressions in both monolayer and SFT. Regarding
222 with the osteocyte markers, the hypoxia non-significantly reduced *Phex*, *Dmp1*, and *Sost* gene

223 expressions in the monolayer, whereas those markers in the SFT were retained by the hypoxia.
224 Hence, we hereby first reported the different manner of *Sost* gene expressions response to
225 hypoxia in the 3D SFT compared to the conventional 2D monolayer condition. We, however,
226 did not evaluate the effect of different oxygen tensions or its duration, so that the effect of
227 various hypoxic condition on the osteocyte differentiation needs to be further studied in the
228 future.

229 While little is known about the hypoxic effect on the osteocyte differentiation *in vitro*, our
230 findings are meaningful because the hypoxia may modulate the initiation and maintenance of
231 osteocyte differentiation in the 3D SFT. Although it is still unclear why the 3D SFT had a
232 response to hypoxia in a different manner compared to the 2D monolayer, these results
233 suggested an importance and requirement of 3D *in vitro* model for fundamental biological
234 research, whereas the conventional 2D model may not fully recapitulate the *in vivo* situation.
235 Since the maintenance of osteocyte-likeness *in vitro* for osteocyte research is still challenging,
236 our 3D model as well as recruitment of hypoxic culture condition has a potential to be utilized
237 as a new *in vitro* culture strategy for the osteocyte.

238 In this present study, we successfully established a fabrication method of SFT reconstructed
239 by mouse osteoblast-like cells based on Furukawa's study[20]. Particularly, the SFT in this study
240 was fabricated by embedding a cell monolayer underneath the SFT to fix it as described in Fig.
241 1(A) and (B). Without the monolayer, the SFT was not able to retain its shape as desired and
242 eventually become shrunken due to their strong cell contraction forces in Fig.1 (B). By using
243 this monolayer embedded underneath, we successfully fabricated the SFT on the culture dish.
244 The SFT is a thick tissue-engineered construct with a thickness of about 50 μm, compared to a
245 conventional cell sheet with a thickness of 20 μm[21].

The SFT developed in this study is also thought to be suitable for *in vivo* study by transplantation for bone regeneration because it is a thicker 3D tissue engineered construct than the conventional cell sheet model[22]. The present method allowed to make it possible to detach the SFT from the normal culture dish by mechanically scrapping it, without recruiting a temperature-responsive culture dish. Since the temperature-responsive culture dish is an essential experimental tool in the conventional cell-sheet technologies[23,24], our novel method did not recruit a specific culture dish to fabricate the SFT. While most of the studies in the bone tissue engineering have utilized a scaffold-based technology for several decades[25], the scaffold is derived from artificial materials which have a risk to cause the foreign body reaction after transplantation. On the other hand, the SFT model does not recruit any artificial materials, but only cells. Hence, the *in vivo* study transplanting the SFT will be expected to become an alternative in the field of tissue engineering for clinical applications.

In conclusion, we successfully established the method to fabricate the SFT reconstructed by mouse osteoblast-like cells. We showed that the osteoblast-like cells in the form of the SFT rendered the osteocyte-likeness within 2 days. Moreover, we first attempted to utilize the 3D SFT, as a new *in vitro* osteocytic model, in response to hypoxia. Our present study suggested that the hypoxia may be required to promote the initiation of osteocyte differentiation and also to maintain the osteocyte differentiation for the osteoblast-like cells *in vivo* situation. Most interestingly, our finding highlighted the potential significance of 3D *in vitro* model to mimic the *in vivo* situation.

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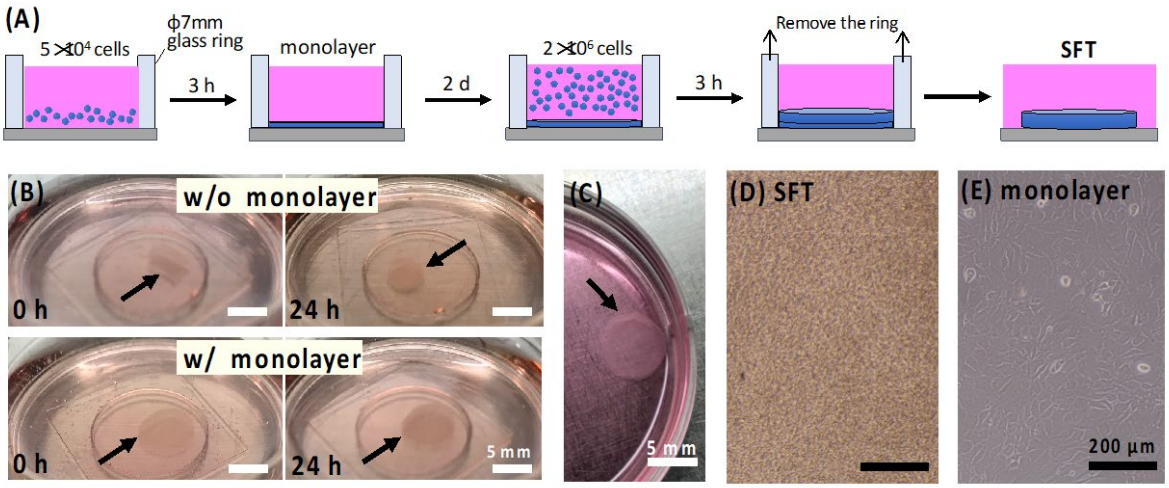

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344

345 Figure 1. Fabrication of scaffold-free osteocytic tissue. (A) Schematic diagram illustrating the
346 fabrication method for scaffold-free tissue (SFT) reconstructed by mouse osteoblast-like cells.
347 (B) Images of SFT with or without a monolayer embedded underneath the SFT immediate or
348 24 hours after removing a glass ring. (C) Image of the SFT after 2-day incubation. Microscope
349 image of (D) scaffold-free tissue and (E) monolayer reconstructed by osteoblast-like cells.

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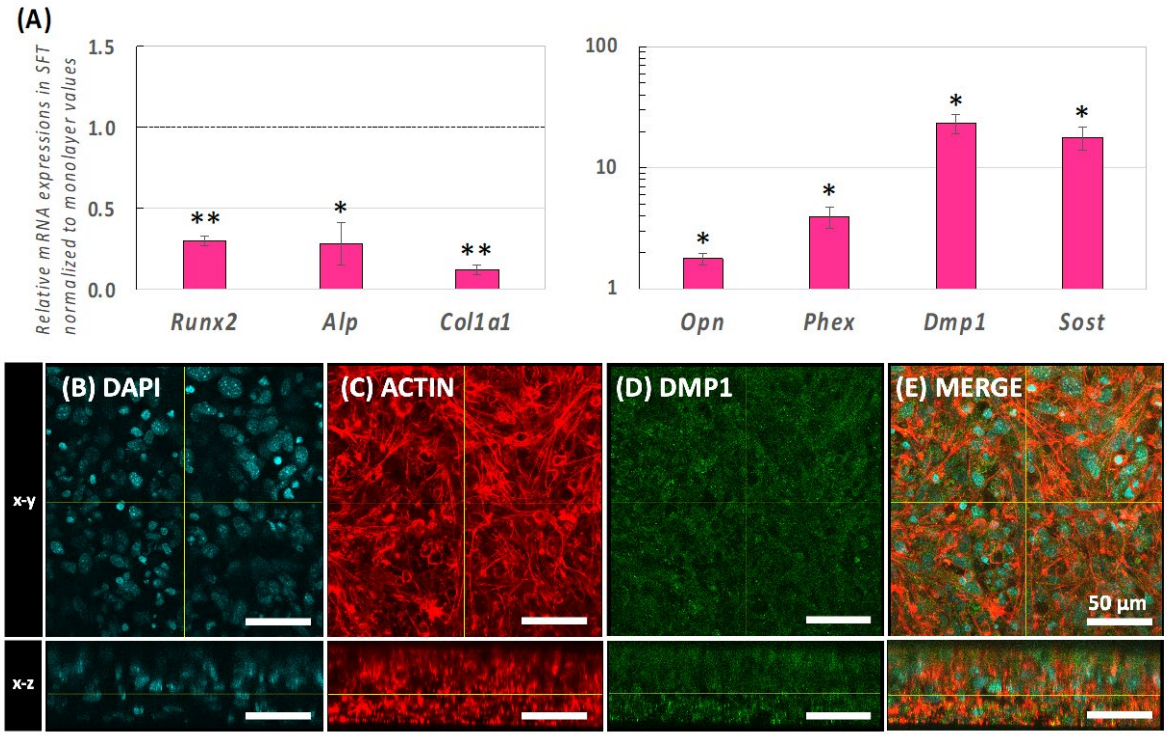

Figure 2. Induced osteocytogenesis in the SFT after 2-day incubation. (A) Real-time PCR analysis of relative mRNA expression of osteoblast markers (*Runx2*, *Alp*, and *Col1a1*) and osteocyte marker (*Opn*, *Phex*, *Dmp1*, and *Sost*) after 2-day incubation. Graphs represent the relative mRNA expression levels in the SFT normalized to *Gapdh* mRNA expression levels and further normalized to monolayer values. The relative mRNA expressions in the SFT were then normalized to that in the monolayer. The bars indicate the mean ± standard error ($n = 7$; p-value was obtained from Student's t-test; * $p < 0.05$, ** $p < 0.005$). Immunostaining images of the scaffold-free tissue, 2-day incubation; (B) DAPI; (C) F-ACTIN; (D) DMP1; (E) MERGE.

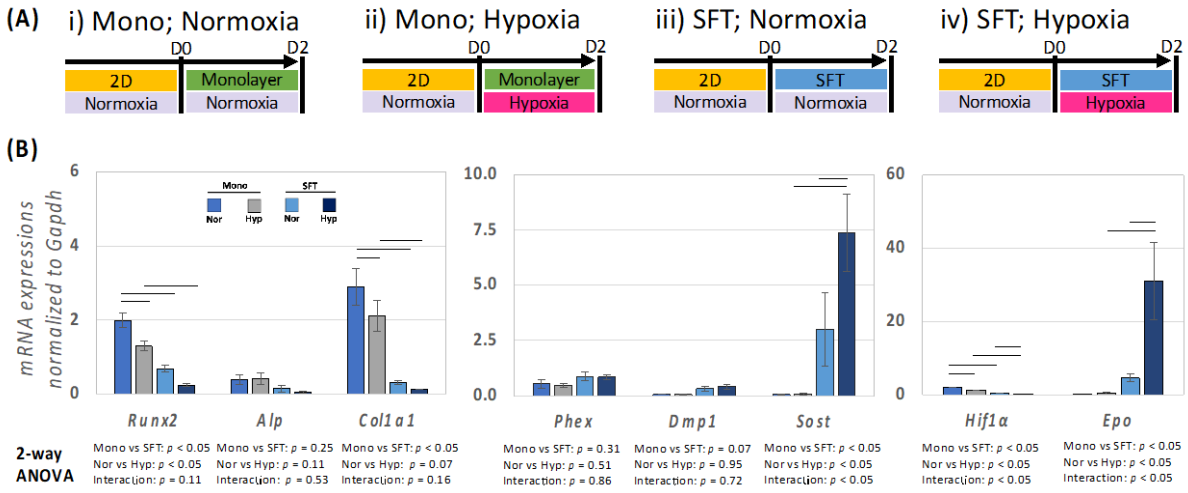

Figure 3. Up-regulation of *Sost* gene expression in the SFT under hypoxia during the fabrication period (Day 0 – 2). (A) Schematic overview of hypoxic experiment for the SFT. (B) The mRNA expression in the monolayer and SFT after applying 5% hypoxia for 48 hours during the fabrication period (Day 0 – 2). Osteoblast (*Runx2*, *Alp*, and *Col1a1*), osteocyte (*Opn*, *Phex*, *Dmp1*, and *Sost*), and hypoxia-induced markers (*Hif1a* and *Epo*) in the monolayer and SFT were measured by real-time PCR, in the absence and presence of hypoxia. The graphs represent the mRNA expression levels normalized to *Gapdh* mRNA expression levels. The colored bars indicate the mean ± standard error (n = 6; bar indicates the significance between the graphs, which was obtained from two-way ANOVA and Fisher's LSD post hoc test; α = 0.05).

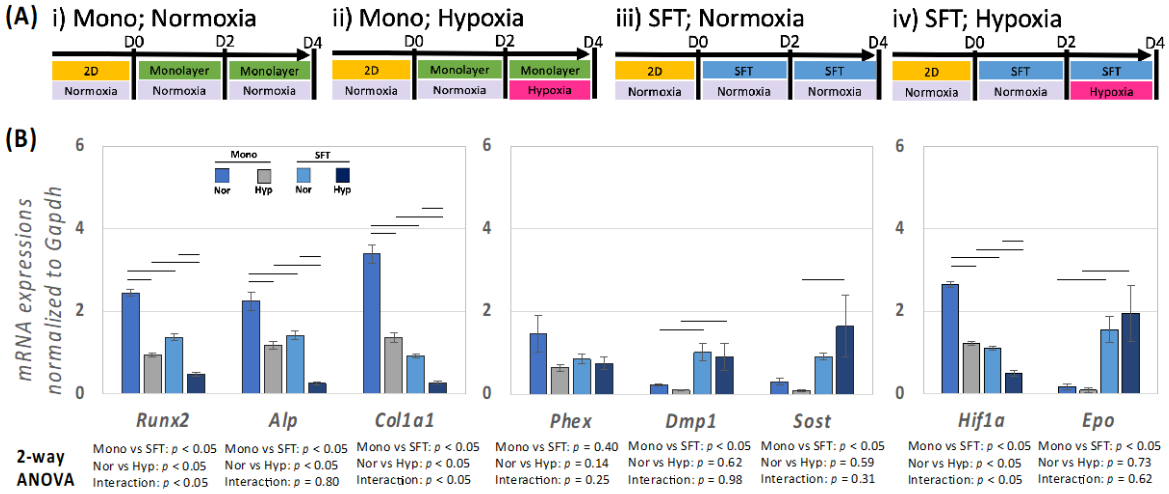

Figure 4. Maintained osteocyte gene expressions in the SFT under hypoxia after the fabrication period (Day 2 – 4). (A) Schematic overview of hypoxic experiment for the SFT. (B) The mRNA expression in the monolayer and SFT being subjected to hypoxic condition for 48 hours after the fabrication period (Day 2 – 4). Osteoblast (*Runx2*, *Alp*, and *Col1a1*), osteocyte (*Opn*, *Phex*, *Dmp1*, and *Sost*), and hypoxia-induced markers (*Hif1a* and *Epo*) in the monolayer and SFT were measured by real-time PCR, in the absence and presence of hypoxia. The graphs represent the mRNA expression levels normalized to *Gapdh* mRNA expression levels. The colored bars indicate the mean ± standard error (n = 6; bar indicates the significance between the graphs, which was obtained from two-way ANOVA and Fisher's LSD post hoc test; α = 0.05).

388 Table 1. Primer List

Gene	Forward primer	Reverse primer	Amplicon size (bp)
Gapdh	TGTTCCTACCCCCAATGTGT	GGTCCTCAGTGTAGCCCAAG	137
Runx2	CAGTCCCAACTTCCTGTGCT	TACCTCTCCGAGGGCTACAA	94
Alp	GCTGATCATTCCCACGTTTT	ACCATATAGGATGGCCGTGA	120
Col1a1	CGTGCAATGCAATGAAGAAC	TCCCTCGACTCCTACATCTTCT	118
Opn	CCCGGTGAAAGTGACTGATT	GGCTTTCATTGGAATTGCTT	191
Phex	AGGCATCACATTCACCAACA	ATGGCACCATTGACCCTAAA	103
Dmp1	GGTTTTGACCTTGTGGGAAA	AATCACCCGTCCTCTCTTCA	91
Sost	CGTGCCTCATCTGCCTACTT	ATAGGGATGGTGGGGAGGT	185
Hif1α	GGCGACACCATCATCTCTCT	TTTGGAGTTTCCGATGAAGG	164
Epo	CCACCCTGCTGCTTTTACTC	GACCTTCTGCACAACCCATC	156

389