The surface topography of an implant fixture is an important factor affecting osseointegration. We herein demonstrated the effects of surface microtopography of titanium disks on proliferation and differentiation of osteoblast-like cells isolated from rat calvariae. Titanium disks with machine surface (MS), rough surface (R1) and rough surface combined with small cavities (R2) were used in an in vitro culture system. Rough surfaces (R1 and R2 disks) induced stronger osteoblast proliferation and differentiation (BGP and sclerostin mRNA expressions and calcium content) than the smooth surface (MS disk). Furthermore, surface microtopography of R2 disk, which was rough with small cavities, more strongly induced cell proliferation and mineralized bone matrix production than R1 disk. Our results suggest that surface microtopography influences osteoblast proliferation and differentiation. R2 disk, which is rough with small cavities, may be used in implant fixtures to increase osseointegration.

**Keywords:** Osteoblast-like cells, Microtopography, Titanium disk, Proliferation, Bone formation

### INTRODUCTION

Integration of titanium implants with bone tissues is an important factor for the prognosis of implant treatments. Secure osseointegration results in clinical satisfaction and contributes to the development of implant treatments. It has been reported that specific characteristics of titanium allow it to provide reliable and immediate osseointegration, e.g., surface roughness\(^1\)–\(^3\), chemistry\(^4\)–\(^5\) and energy\(^6\)–\(^7\). Among these characteristics, surface roughness results in a significant clinical advantage because the structure directly influences both bone modeling and remodeling in the bone tissues without negative side effects. In fact, several studies reported that the surface topography of titanium enhanced bone-implant contact, osteoblasts differentiation\(^8\)–\(^9\) and local factor production\(^10\). These results indicate that modification of surface structures influence osteoblast proliferation and differentiation and growth factor production. However, some studies reported that surface microtopography of titanium had no effect on osteoblast functions\(^11\) and decreased osteoblast proliferation\(^12\). Therefore, mechanisms of osteoblast functions influenced by surface microtopography have remained unclear.

The surfaces of cancellous and cortical bone contain rough structures consistent with shallow pits (Hawship’s lacuna) formed by osteoclasts, and osteoblasts later secrete new bone matrix to fill in the pits. The cycle of bone resorption by osteoclasts and bone formation by osteoblasts is repeated in skeletal tissue and referred to as bone remodeling\(^13\). Given that bone surface roughness is important for bone formation by osteoblasts, it has been postulated that roughness of titanium implants also induces osteoblast functions leading to osseointegration. To confirm this hypothesis, we examined the effects of titanium disks with various degrees of roughness on osteoblast proliferation and differentiation using osteoblast-like cells isolated from rat calvariae.

### MATERIALS AND METHODS

**Titanium disks**

Titanium disks measuring 20 mm in diameter and 1-mm thick were made from sheets of grade 4 unalloyed titanium (ASTM F67 N: \(\leq 0.05\)%, C: \(\leq 0.08\)%, H: \(\leq 0.015\)%, Fe: \(\leq 0.50\)%), O: \(\leq 0.40\)%, Ti: Balance) and supplied by GC (Tokyo, Japan). These disks possessed three different surface roughness. The machine surface (MS) was a smooth surface, and two sandblasted surfaces were obtained by different abrasive particles: R1 (45 \(\mu\)m diameter, \(MgO\) and etching with 1 N HCl) and R2 (200 \(\mu\)m diameter, \(Al_2O_3\) and etching with 8 N \(H_2SO_4\) and 5 N HCl). All disks were carefully washed in distilled water, rinsed with 80% ethanol, cleaned in absolute alcohol and air dried. The disks were then sterilized by gamma radiation.
Roughness assessment
The titanium surfaces (n=5 in each surface condition) were analyzed by a roughness tester (Surftest 470A, Tokyo Seimitsu, Tokyo, Japan). Five linear tracts 2.5 mm in length and cutoff 0.3 mm/s were evaluated on each disk. We used three different parameters to evaluate surface roughness: Ra, arithmetic average of the absolute values of all points of the profile (µm); Rz, arithmetic average of the maximum peak-to-valley height of the ten greatest values (µm); and Rt, range of roughness, the maximum peak-to-valley height (µm).

Cell culture
Animal experiments were performed under a protocol approved by the animal care unit under the authority of Ohu University, Fukushima, Japan (No. 201127). Osteoblast-like cells were isolated from calvaria of 1-day-old Sprague Dawley rats by sequential enzymatic digestion as described previously by Yokose et al. Briefly, calvariae were incubated at room temperature for 20 min with gentle shaking in an enzyme solution containing 0.1% collagenase, 0.05% trypsin and 4 mmol/L EDTA in calcium- and magnesium-free phosphate-buffered saline (PBS). This procedure was repeated to yield a total of six digests. The cells retrieved from the last four to six digests were separately plated in a T-75 flask (Falcon Labware, Lincoln Park, NJ, USA) with α-Minimum Essential medium (α-MEM) containing 10% calf serum and antibiotics (100 IU/mL of penicillin G and 100 µg/mL of streptomycin). After reaching a subconfluent state, the cells were removed from each flask and cultured with the same medium. The second passage of the cells was used for all subsequent experiments as described below. The cells were plated onto each titanium disk at a density of 2x10^5/cm² and cultured in α-MEM containing 10% calf serum, 300 µg/mL β-glycerophosphate, 50 µg/mL ascorbic acid and antibiotics (mineralizing medium). The medium was changed every 2 days, and the cells were incubated up to 28 days.

Cell proliferation
Cell proliferation of osteoblast-like cells on each titanium disk after 5, 7 and 14 days in culture was measured using a cell counting kit (Wako Pure Chemical Industries, Osaka, Japan) according to the manufacturer's protocol.

Real-time quantitative PCR
Total RNA was extracted from the cells and calvaria tissues using the EASY Prep Plus RNA kit (Takara Biomedical, Shiga, Japan) and treated with DNase I (Takara Biomedical). cDNA was synthesized from total RNA (1 µg) using an RT-PCR kit (Takara Biomedical) and random primers. Subsequently, 100 ng cDNA was used as a template for the second step of real-time PCR. Primers were used at 5 µM with 12.5 µL SYBR Green Premix (Takara Biomedical) in a final volume of 25 µL. SYBR Green PCR amplification and real-time fluorescence detection were performed using the Smart Cycler II System (Takara Biomedical). Rapid PCR cycling conditions were as follows: 95°C for 10 s (denaturation), followed by 45 cycles at 95°C for 5 s and 60°C for 20 s (annealing/extension). Primers were purchased from Takara Biomedical and the sequences were as follows: β-actin: 5'-TGACAGGATGCAAGAAGGAGA-3' and 5'-TAGAGCCACCAATCCACAC-3'; Bone gla protein (BGP): 5'-AGACTCGGCGACCTCAG-3' and 5'-CGTCCTGGAAGCAATGTG-3'; and sclerostin: 5'-AGCTTCAAGATGATGCTCAC-3' and 5'-AACGGGTTGTAGTGCAGCCTC-3'. Measurements were taken during the 60°C extension step in each cycle, and the second-derivative method was used to calculate the threshold cycle. The target gene expression level was normalized to that of β-actin in each sample. After amplification, melting curve analysis of the PCR products was used to differentiate between specific and non-specific PCR products.

BGP mRNA expression were examined on day 21, and sclerostin mRNA expression was examined on day 28.

Calcium concentration
Osteoblast-like cells on each titanium disk were rinsed with PBS and treated with 500 µL of 4 N HCl for 12 h at room temperature. After centrifugation for 10 min at 6,000 g, the calcium concentration in the supernatants was measured.

Scanning electron microscopy (SEM)
To examine surface microtopography and cell attachment to the disk surfaces (three surface conditions), specimens were observed by SEM (N-3500S, Hitachi, Tokyo, Japan) after fixation in 10% formalin and dehydration with ethanol (at 25, 50, 70 and 100% ethanol concentrations for 5, 5, 5 and 10 min, respectively). The specimens were then critical point dried with 100% tert-butyl alcohol for 5 min, followed by sputter coating with palladium for 90 s (Ion sputter 101E, Hitachi) and coated with gold. The specimens were observed by SEM (N-3500S, Hitachi, Tokyo, Japan). Five linear tracts were analyzed by a roughness tester (Surftest 470A, Tokyo Seimitsu, Tokyo, Japan). The titanium surfaces were analyzed by a roughness tester (Surftest 470A, Tokyo Seimitsu, Tokyo, Japan). Five linear tracts 2.5 mm in length and cutoff 0.3 mm/s were evaluated on each disk. We used three different parameters to evaluate surface roughness: Ra, arithmetic average of the absolute values of all points of the profile (µm); Rz, arithmetic average of the maximum peak-to-valley height of the ten greatest values (µm); and Rt, range of roughness, the maximum peak-to-valley height (µm).

Statistical analysis
Data are presented as the mean±standard deviation (SD). The data were analyzed by the Kruskal-Wallis H-test, and differences in the means were assessed using the Mann-Whitney U-test with Bonferroni correction. Values less than p=0.05 were considered as statistically significant.

RESULTS
Surface characterization
Figure 1 and Table 1 demonstrate the surface structure of the sample disks. MS disk was the least rough, while R2 disk was the roughest (Table 1). R2 disk surface topography also demonstrated many irregular cavities (asterisks in Fig. 1), with a diameter and depth of
Fig. 1 Upper panel demonstrates the gross appearance of each titanium disk surface, and middle panel shows SEM images of each disk surface. Small cavities (*) can be seen on R2 disk. Lower panel shows laser microscope 3D images of each disk surface.

<table>
<thead>
<tr>
<th>Roughness parameter (µm)</th>
<th>MS</th>
<th>R1</th>
<th>R2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ra</td>
<td>0.29±0.05</td>
<td>1.27±0.18</td>
<td>3.26±0.11</td>
</tr>
<tr>
<td>Rz</td>
<td>1.61±0.23</td>
<td>10.21±1.10</td>
<td>19.22±1.25</td>
</tr>
<tr>
<td>Rt</td>
<td>2.01±0.26</td>
<td>12.94±1.67</td>
<td>21.92±2.14</td>
</tr>
</tbody>
</table>

Ra: arithmetic average of the absolute values of all points of the profile; Rz: arithmetic average of the maximum peak-to-valley height to the ten greatest values; Rt: the maximum peak-to-valley height of the entire measurement trace.

approximately 50 and 21 µm, respectively.

Osteoblast growth on the disks

Osteoblast growth on each disk after 5 days in culture is shown in Fig. 2. A small number of cells were scattered on MS disk (Fig. 2a). A large number of cells covered the surfaces of R1 and R2 disks (Figs. 2b and c).

The change in cell number over time is shown in Fig. 3. The number of cells increased over time with all disks. On day 5 in culture, the greatest number of cells was observed on R2 disk, followed by R1 disk. On day 7, the greatest number of cells was observed on R2 disk, however, no significant difference in the number of cells grown on R1 and MS disks was observed. The cells grown on all disks reached confluence on day 14 in culture.

The cell characteristics reflected terminal differentiation of osteoblasts on day 21 in culture. BGP mRNA expression, which is a marker of osteoblast terminal differentiation, was observed in all experimental cells. The cells grown on R2 disk demonstrated significantly higher BGP mRNA expression levels than those grown on R1 and MS disks (p<0.05). Additionally, the cells grown on R1 disk showed significantly higher BGP mRNA expression levels than those grown on MS disk (p<0.05) (Fig. 4). Similarly, calcium concentration was significantly increased in the
cells grown on R2 disk compared to those grown on R1 and MS disks ($p<0.05$), and calcium concentration in the cells grown on R1 disk was significantly increased compared to those grown on MS disk ($p<0.05$) (Fig. 5). The cells were cultured up to 28 days in calcification medium and sclerosis mRNA expression levels were determined. Although sclerostin mRNA expression, which is marker of mature osteocyte$^{16}$, was observed in all experimental cells, the cells grown on R2 disk showed the strongest expression ($p<0.05$) (Fig. 6).

DISCUSSION

As undifferentiated mesenchymal cells isolated from rat calvariae can be differentiated into mature osteoblastic cells in culture systems$^{15}$, this can be a useful tool to examine osteoblast functions. We previously demonstrated that osteoblast differentiation can be accelerated by BMPs$^{17}$, PTH$^{18}$, interleukin (IL)-6$^{19}$, IL-1 and IGF-1$^{14}$. It is well known that growth factors induce strong biological stimulation and are available for clinical application, particularly implant treatments. However, these molecules may also induce unintentional effects in untargeted tissues and organs. Osteoblast differentiation can be influenced by many factors in addition to growth factors, such as scaffold containing matrix protein$^{20,21}$ and 3-dimensional (3D) surface properties$^{1-3,6-12}$. Although much research has focused on the effects of surface microtopography of titanium on
osseointegration, little information regarding the precise biological mechanism of osteoblasts was presented. For accurate prognosis of implant treatments, detailed analysis of osteoblast proliferation and differentiation during osseointegration procedures on titanium surfaces is needed.

For these reasons, in this study we focused on surface microtopography of titanium and examined the effects of surface structures on osteoblast differentiation using an in vitro culture system and titanium disks with various degrees of roughness. We evaluated disks with a smooth surface (MS), a rough surface (R1) and a rough surface with dents with diameters ranging 50–100 µm (R2). Our results indicated that rough surfaces (R1 and R2 disks) influenced osteoblast proliferation and differentiation. Guizzardi et al. demonstrated that the attachment of cell membrane to a scaffold is indispensable for osteoblast proliferation and differentiation, and osteoblasts cultured on a rough surface had increased alkaline phosphatase production. Our findings support their results and the rough surfaces of the titanium disk could present a suitable environment for osteoblast activities. In particular, osteoblasts on R2 disk formed mineralized bone matrix and were embedded in the matrix. Furthermore, microtopography composed of small cavities on the rough surface of R2 disk stimulated increased cell growth, BGP mRNA expression and calcium concentration than the other disks. By day 28, the cells grown on R2 disk expressed the highest sclerostin mRNA expression levels of all experimental cells. Sclerostin is expressed in mature osteocyte embedded in mineralized matrix in bone tissue. Consequently, the cells grown on R2 disk were embedded in mineralized matrix and fully differentiated in mature osteocytes. This indicated that microtopography of R2 disk induced mature bone tissue growth on the surface. In normal bone tissues, bone remodeling is carried out in all bones, and remodeling can be initiated by bone resorption with osteoclasts. Osteoblasts then form new bone matrix in shallow pits created by osteoclasts, which are approximately 50 µm in depth. We speculate that the cavities on R2 disk, which had diameters ranging 50–100 µm, may resemble the pits formed by osteoclasts, presenting a suitable environment for osteoblast differentiation during bone remodeling. In addition, there is the possibility that not only small cavities surface but also the difference of roughness structure between R1 and R2 may influence the osteoblast proliferation and differentiation.

In this study, we did not evaluate the expression of early markers of osteoblast differentiation, such as alkaline phosphatase and/or type I collagen. In clinical implant treatment, the most important factor may be osseointegration. For this reason, we focused on cell proliferation and bone formation on titanium disks and examined the cell number and terminal markers of osteoblasts, such as BGP and sclerostin mRNA expressions and calcium concentration. However, biological effects of microtopography on osteoblast proliferation and differentiation may be dependent on attachment of the cell membrane to the titanium surface. In fact, Chang et al. demonstrated that fibronectin and integrin could be important factors for osteoblast differentiation. Future investigations should focus on the mechanism by which cells attach to the titanium surface.
CONCLUSION
Our results indicated that titanium with rough surfaces containing small cavities can present a suitable environment for osteoblast proliferation and differentiation. This type of disk can be advantageous for osseointegration of implant treatments in clinical practice.

CONFLICT OF INTEREST
The authors declare that they have no conflict of interest.

REFERENCES