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Strontium and magnesium ions released from bioactive titanium metal promote early bone bonding in a rabbit implant model

Yaichiro Okuzu [a,*], Shunsuke Fujibayashi [a], Seiji Yamaguchi [b], Koji Yamamoto [c], Takayoshi Shimizu [a], Takashi Sono [a], Koji Goto [a], Bungo Otsuki [a], Tomiharu Matsushita [b], Tadashi Kokubo [b], Shuichi Matsuda [a]

[a] Department of Orthopaedic Surgery, Kyoto University Graduate School of Medicine, 54 Shogoin-Kawaharacho, Sakyo-ku, Kyoto 606-8507, Japan
[b] Department of Biomedical Sciences, College of Life and Health Sciences, Chubu University, 1200 Matsumoto-cho, Kasugai, Aichi, 487-8501, Japan
[c] Department of Biomedical Engineering, Faculty of Life and Medical Sciences, Doshisha University, 1-3 Tatara Miyakodani, Kyotanabe-shi, Kyoto 610-0394, Japan

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ABSTRACT

We have previously developed the "alkali and heat treatment" method to confer bioactivity (bone-bonding ability) to titanium metal (Ti). As strontium (Sr) and magnesium (Mg) ions reportedly promote osteoblastic cell proliferation and differentiation and accelerate bone formation, we improved this method to induce the release of Sr (Sr-Ti) or Mg (Mg-Ti) ions from Ti in a previous study. Here, we evaluated the bioactivity of these novel surface treatments, Sr-Ti and Mg-Ti. *In vitro* evaluation of cell viability, expression of *integrin β1*, *β catenin*, and *cyclin D1*, osteogenic gene expression, alkaline phosphatase activity, and extracellular mineralization using MC3T3-E1 cells revealed that Sr-Ti and Mg-Ti enhanced proliferation and osteogenic differentiation. In rabbit *in vivo* studies, Sr-Ti and Mg-Ti also provided greater biomechanical strength and bone-implant contact than the positive control Ti (Ca-Ti), especially at the early stage (4–8 weeks), and maintained these properties for a longer period (16–24 weeks). Advantages of the improved method include process simplicity, applicability for any implant shape, and lack of adverse effects on implant composition and structure. Therefore, our treatment is promising for clinical applications to achieve early bone bonding.

Statement of Significance

Implantation into osteoporotic bone constitutes a challenging problem because of early migration or loosening of the implant, which is primarily due to insufficient initial fixation in porotic bone. Therefore, it is desirable to provide implants with a capacity for early bone bonding. We have achieved conferring early bone bonding ability to titanium metal by releasing strontium ions or magnesium ions. Our treatment is promising for clinical applications to achieve early bone bonding of orthopedic or dental Ti-based implants.

1. Introduction

Instrumentation failure such as loosening of implants [1] is a major problem in orthopedic and dental surgery, although various surface treatments and micro- or nano-structure technologies have recently improved the implants available. Among various methods providing implants with bone-bonding capability [2,3], hydroxyapatite (HA) coating remains the most biocompatible and widespread, and has achieved a certain degree of success. However, HA coating degrades on the long term [4].

We have reported a novel surface treatment, that is, heat treatment after soaking in NaOH or HCl solution, which resulted in the formation of a uniform bioactive layer on titanium metal (Ti) [5–7]. Bone-like apatite layer formed on the surface in a physiological environment and Ti was able to bond directly to living bone through this layer. This "alkali and heat treatment" has been applied to the porous Ti surface of total hip arthroplasty prosthetics, with a product commercialized in Japan in 2007 [8]. Further, we developed Ca-treated Ti, in which soaking in $CaCl_2$ solution and ultrapure water was added to the process to achieve surface stability, because in alkali and heat treatment, the apatite-forming ability on the surface layer may deteriorate in high-humidity environments [9]. The bioactivity of Ca-treated Ti was comparable to that of alkali- and heat-treated Ti [9,10].

* Corresponding author.
E-mail address: yokuzu@kuhp.kyoto-u.ac.jp (Y. Okuzu).

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Table 1
Cp-Ti and three types of surface treatment used in this study.

Sample	Pretreatment	1st step	2nd step	3rd step	4th step
Cp-Ti	Done	None	None	None	None
Ca-Ti	Done	5 M NaOH; 60 °C; 24 h	100 mM $CaCl_2$; 40 °C; 24 h	600 °C; 1 h	H_2O; 80 °C; 24 h
Sr-Ti	Done	5 M NaOH; 60 °C; 24 h	50 mM $CaCl_2$ + 50 mM $SrCl_2$; 40 °C; 24 h	600 °C; 1 h	1 M $SrCl_2$; 80 °C; 24 h
Mg-Ti	Done	5 M NaOH; 60 °C; 24 h	40 mM $CaCl_2$ + 60 mM $MgCl_2$; 40 °C; 24 h	600 °C; 1 h	1 M $MgCl_2$; 80 °C; 24 h

Pretreatment: washes with acetone, 2-propanol, and ultrapure water in an ultrasonic cleaner for 30 min each followed by drying at 40 °C.

In orthopedics, osteoporosis and the associated fragility fractures are important concerns because of the increasing number of individuals affected, cost of treatment, and difficulty in activities of daily living. Implantation into osteoporotic bone also constitutes a challenging problem because of early migration or loosening of the implant, which is primarily due to insufficient initial fixation in such bone [11,12]. Therefore, implants with a capacity for earlier bone bonding are desirable.

Strontium (Sr) has been used as anti-osteoporotic agent in the form of strontium ranelate [13]. In particular, strontium ranelate has a dissociating effect on bone remodeling by maintaining bone formation and decreasing bone resorption [14]. This effect was also reported in an *in vitro* study, in which Sr ions promoted osteoblast proliferation and differentiation, and reduced osteoclast differentiation, activity, and bone resorption [15]. However, it has been reported that systemic administration of strontium ranelate might increase the rate of cardiovascular events [16]. Therefore, localized Sr action is desirable to avoid side effects. Studies of implants containing or releasing Sr ions have also reported enhanced implant osseointegration or improved bioactivity [17,18]. Similarly, magnesium (Mg) ions reportedly promote osteoblastic differentiation in bone marrow stromal cells (BMSCs) [19,20]. Furthermore, implants incorporating Mg showed higher osteoblast proliferation and differentiation [21,22].

Considering these advantages, we improved the alkali and heat treatment method to include Sr or Mg in the Ti surface and to allow the release of these ions for obtaining earlier bone-bonding [23,24]. Notably, Ti treated with this method showed an apatite layer on its surface in a physiological environment. In the current study, we evaluated the *in vitro* and *in vivo* bioactivities of Ti implants releasing Sr ions or Mg ions with this novel surface treatment.

2. Materials and methods

2.1. Sample preparation

Commercially pure Ti (cp-Ti; Ti > 99.5%) purchased from Nilaco (Japan) was washed with acetone, 2-propanol, and ultrapure water in an ultrasonic cleaner for 30 min each and then dried at 40 °C. After cleaning, we prepared surface-treated Ti with Ca (Ca-Ti), Sr (Sr-Ti), or Mg (Mg-Ti) as previously described [9,23,24]. Briefly, cp-Ti was initially treated with 5 M NaOH. For subsequent Ca treatment, the metal was soaked in 100 mM $CaCl_2$ solution at 40 °C for 24 h, exposed to 600 °C for 1 h, and finally soaked in ultrapure water at 80 °C for 24 h. For Sr and Mg treatments, the metal was soaked in 50 mM $CaCl_2$ + 50 mM $SrCl_2$ and 40 mM $CaCl_2$ + 60 mM $MgCl_2$, respectively, at 40 °C for 24 h, exposed to 600 °C for 1 h, and treated with 1 M $SrCl_2$ and 1 M $MgCl_2$, respectively (Table 1.). The four types of Ti samples were sterilized with ethylene oxide gas.

2.2. In vitro study

2.2.1. XTT cell viability assay

MC3T3-E1 cells were seeded onto cp-Ti, Ca-Ti, Sr-Ti, and Mg-Ti disk specimens in 12-well plates at 2×10^4 cells/well and cultured in α-MEM (Gibco, USA) with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37 °C in a humidified atmosphere of 5% CO_2. After 1, 3, and 5 days, XTT (Roche Applied Sciences, USA) was added for 4 h. Then, 150 μl of the medium was transferred to a 96-well plate and the formazan product was quantified by measuring the absorbance at 450 nm using a Multiskan JX (Thermo Labsystems, USA) [25]. In this experiment, four disk specimens including different lots were prepared for each of the four types of Ti samples.

2.2.2. Quantitative reverse-transcription (RT-q) PCR

MC3T3-E1 cells were seeded onto the four samples in 12-well plates at 5×10^4 cells/well as described above, and cultured for 3 or 7 days. Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Germany) following the manufacturer's protocol, and reverse-transcribed with ReverTra Ace qPCR Master Mix (Toyobo, Japan). RT-qPCR was carried out on the LightCycler system (Roche) with Thunderbird SYBR qPCR Mix (Toyobo). Primer sequences are listed in Table 2. Gene expression levels of *β-catenin*, *integrin β1*, *cyclin D1*, and osteogenic differentiation-related genes (*Runx2*, *Alp*, *Ocn*, and *Opn*) were normalized to those of *Gapdh*. These experiments were performed using five disk specimens including different lots for each of the four Ti samples.

2.2.3. Immunofluorescence staining of integrin β1 to evaluate expression and localization

MC3T3-E1 cells were cultured on samples in 12-well plates as described for 24 h, washed three times with PBS, and fixed with 4% paraformaldehyde for 12 min and with 0.2% Triton X-100 for 10 min. Samples were blocked with 4% FBS for 30 min, incubated with an antibody targeting integrin β1 (ab95623; Abcam, UK) diluted in 1% FBS and Tris-buffered saline with Tween 20 (TBST) at room temperature for 1 h, washed three times with TBST, and incubated with DyLight 488-conjugated anti-rat IgG antibody (ab169346; Abcam) for 1 h in the dark. Rhodamine-phalloidin antibody was applied for 20 min followed by DAPI for 5 min in the dark. After washing with TBST and distilled water, the samples

Table 2
Sequence and target gene information for primers used for RT-PCR in the current study.

Gene	Primer sequence (F, forward; R, reverse; 5′-3′)
Gapdh	F: TGTCCGTCGTGGATCTGAC
	R: TGTCCGTCGTGGATCTGAC
β catenin	F: GCCTACCACCAGCAGAATGT
	R: GAGGTGGCTGGGACTGTG
Integrin β1	F: TTGGGATGATGTCGGGAC
	R: AATGTTTCAGTGCAGAGCC
Cyclin D1	F: TTTCTTTCCAGAGTCATCAAGTGT
	R: TGACTCCAGAAGGGCTTCAA
Runx2	F: CCACAAGGACAGAGTCAGATTACA
	R: TGGCTCAGATAGGAGGGGTA
Alp	F: ACTCAGGGCAATGAGGTCAC
	R: CACCCGAGTGGTAGTCACAA
Ocn	F: AGACTCCGGCGCTACCTT
	R: CTCGTCACAAGCAGGGTTAAG
Opn	F: GGAGGAAACCAGCCAAGG
	R: TGCCAGAATCAGTCACTTTCAC

were mounted and observed using fluorescence microscopy (BZ-X710, Keyence, USA).

2.2.4. Alkaline phosphatase (ALP) activity assay to evaluate cellular differentiation

MC3T3-E1 cells seeded onto the samples in 12-well plates as described above were cultured for 7 days. The cells were then washed twice with normal saline and lysed with 1% NP-40 by extensive pipetting. After incubation with 6.7 mM *p*-nitrophenyl phosphate (Wako Pure Chemical Industries, Japan) at 37 °C for 30 min, the optical density at 405 nm was measured and ALP activity was calculated by extrapolation from a standard curve. ALP activity was normalized against total protein content. These experiments were performed using six disk specimens including different lots for each of the four Ti samples.

2.2.5. Evaluation of extracellular matrix (ECM) mineralization by Alizarin red staining

MC3T3-E1 cells were cultured on samples in 12-well plates as described for 24 h. Then, the medium was changed to an osteogenic medium containing 10 mM β-glycerol phosphate and 50 μg/l ascorbic acid (both from Sigma-Aldrich, USA). After a 3-week culture, the cells were washed twice with phosphate-buffered saline (PBS), fixed in 100% methanol for 20 min at 4 °C, and washed with distilled water twice. The cells were then stained with 40 mM Alizarin Red for 10 min in the dark, and washed with distilled water repeatedly. After photographs of the samples were taken, the samples were dried. For quantitation, stained calcium nodules were dissolved in 10% cetylpyridinium chloride solution and the optical density at 540 nm was measured. The values were expressed relative to that of cp-Ti. We performed this experiment with five disk specimens including different lots for each of the four types of Ti samples.

2.3. In vivo study

2.3.1. Surgical procedure

We utilized 72 mature male Japanese white rabbits (weight: 2.8–3.5 kg) in this study. This study was approved by the Animal Research Committee, Graduate School of Medicine, Kyoto University, Japan (approval number: Med Kyo 17223). We used the four types of Ti samples; cp-Ti, Ca-Ti, Sr-Ti, and Mg-Ti (2 mm × 10 mm × 15 mm; Fig. 1a). Surgical procedures were previously described [26]. Briefly, rabbits were anesthetized by intravenous injection of pentobarbital sodium (40 mg/kg), inhaled isoflurane, and local administration of 1% lidocaine solution. A 3-cm longitudinal skin incision was made on the medial side of the proximal tibia. The fascia and the periosteum were incised and retracted to expose the tibial cortex. A slit-like perforation of 16 mm × 2 mm was made using a dental burr from the medial to the lateral cortex parallel to the longitudinal axis of the tibia. After the hole was irrigated with saline, each sample was implanted (Fig. 1b and c). After saline irrigation, the fascia and the skin were sutured layer by layer. The rabbits were housed individually in standard cages and fed standard rabbit food and water.

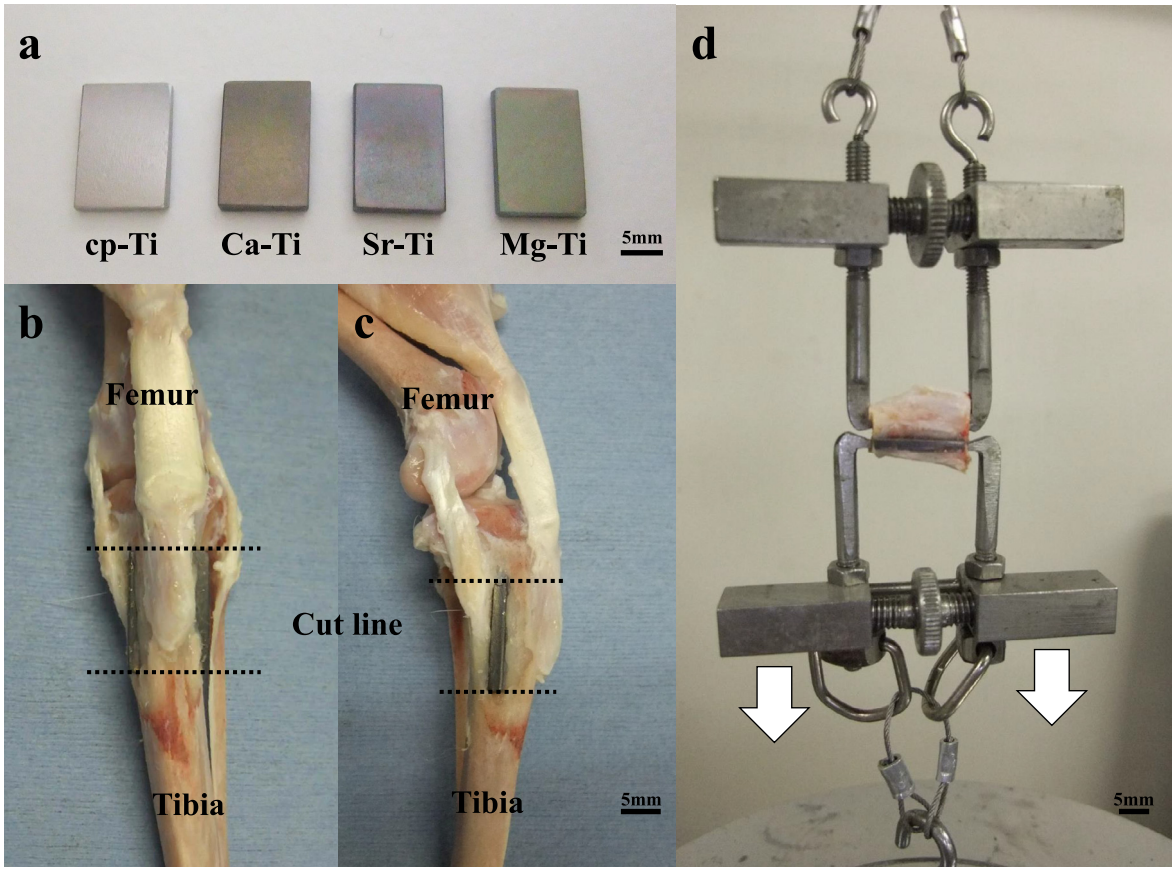

Fig. 1. Sample appearance, implantation into the tibia, and the biomechanical test used in the *in vivo* study. (a) Cp-Ti and surface-treated Ti plates. (b, c) Representative images of the knee after sample explantation. (d) Detachment test to measure the bone-bonding strength of the implant.

At 4, 8, 16, and 24 weeks post operation, 18 rabbits were sacrificed with an overdose of intravenous pentobarbital sodium for biomechanical testing and histological evaluation. Following euthanasia, segments of the proximal tibia containing the implanted samples were cut at the proximal and distal edges of the implant. All specimens were kept moist after harvesting. Of the 36 legs of 18 the rabbits at each time point, 3 legs were assigned to the cp-Ti group and 6 legs to each treated Ti sample group for biomechanical testing, and 3 legs to the cp-Ti group and 4 legs to each treated Ti sample group for histological evaluation.

2.3.2. Biomechanical testing by the detachment test

The detachment test was performed within a few hours from explantation to evaluate the bone-bonding strength of each sample as previously described [26]. Traction was applied vertically to the implant surface at 35 mm/min using an Instron-type autograph (model 1011; Aikoh Engineering, Japan) (Fig. 1d). Detachment failure load was measured when the sample plate detached from the bone. If the plate detached before the test, the failure load was defined as 0 N.

2.3.3. Histology

Explanted specimens were fixed in phosphate-buffered 10% formalin for 10 days, dehydrated in 70%, 80%, 90%, 99%, and 100% [v/v] ethanol for 3 days at each concentration, and embedded in polyester resin. Sections (500 μm) were cut with a band saw (BS-3000CP; Exakt Apparatebau GmbH, Germany) perpendicular to the tibial axis and ground to a thickness of 50–60 μm using a Micro-grinding MG-4000 (Exakt Apparatebau GmbH). Each section was stained with Stevenel's blue/van Gieson's picrofuchsin to stain calcified bone bright red and soft tissue blue. The sections were analyzed under a transmitted light microscope (Eclipse 80i; Nikon, Japan) with a digital camera (DS-55M-L1; Nikon) and subjected to quantitative histomorphometry to determine the amount of direct bone-implant contact (BIC) using Image J (National Institutes of Health, USA).

2.3.4. Serum concentration of Sr and Mg ions

Blood samples of the rabbits with Sr-Ti or Mg-Ti implants (approximately 2 ml) were collected at 1 day, 3 days, and 1, 2, 4, 8, 16, and 24 weeks postoperatively. The samples were centrifuged at 1500*g* for 5 min, and the sera were collected into sample tubes. Serum Sr and Mg ion concentrations were measured by inductively coupled plasma emission spectroscopy (SPS3100; Seiko Instruments, Japan).

2.3.5. Sr and Mg content analyses in bone

To evaluate whether the released Sr and Mg was contained in the bone near the Sr-Ti and Mg-Ti implants, we analyzed thick specimen sections at 4 and 8 weeks using X-ray photo-electron spectroscopy (PHI 5000 VersaProbe II; Ulvac, Japan). Narrow scan spectra of Sr3s and Mg1s were collected with an Mg Kα radiation line as the X-ray source. The C1s peak at 284.8 eV was used for charge correction. Measurements were done at the implant, bone near the implant, and bone far from the implant.

2.4. Statistical analysis

All data are shown as means ± standard deviations. Significant differences among sample groups were determined using one-way analysis of variance (ANOVA) followed by Tukey's HDS tests. Serum ion concentrations were evaluated using Dunnett's test, with preoperative concentration defined as a control. $p < 0.05$ was considered statistically significant. All analyses were performed using JMP Pro 11.0.0 (SAS Institute, USA).

3. Results

3.1. In vitro study

As indicated by the XTT assay, which reflects cell proliferation and cellular metabolism, the three treated samples had no cytotoxic effects, and significantly higher cell viability was detected for Mg-Ti at 1, 3, and 5 days (Fig. 2a). Next, we examined the expression of *β-catenin*, which is a key intracellular factor in Wnt/β-catenin signaling, one of the major osteoblast differentiation signal pathways, of *integrin β1*, which is a key factor involved in cell adhesion, and of *cyclin D1*, which is closely involved in cell proliferation. Sr-Ti induced significantly higher *β-catenin* expression at day 3 (Fig. 2b). Sr-Ti and Mg-Ti induced significantly increased *integrin β1* expression at 3 days and 7 days, respectively (Fig. 2c). Furthermore, Sr-Ti and Mg-Ti induced significantly higher *cyclin D1* expression at day 7 (Fig. 2d). Immunofluorescence showed that cells spread extensively, and integrin β1 expression appeared to be higher and more widely distributed on Sr-Ti and Mg-Ti (Fig. 2e).

RT-qPCR to assess the expression of osteogenic differentiation-related genes (*Runx2*, *Alp*, *Ocn*, and *Opn*) indicated almost no significant differences among the four samples at 3 days (Fig. 3a–d), although Sr-Ti and Mg-Ti induced significantly higher expression at 7 days than cp-Ti. ALP activity was consistent with *Alp* gene expression (Fig. 4a). Furthermore, Sr-Ti induced higher ECM mineralization than cp-Ti and Ca-Ti as indicated by Alizarin Red staining (Fig. 4b). Thus, Sr-Ti and Mg-Ti showed higher osteoblast differentiation ability than cp-Ti and Ca-Ti.

3.2. In vivo study

The surgical procedures were uneventful in all rabbits; no infection, implant dislocation, or adverse reactions, such as inflammation or foreign body reactions, were observed.

The bone-bonding strength between implant and bone was evaluated using the detachment test. At 4 weeks, Mg-Ti induced significantly higher failure load than cp-Ti and Ca-Ti (Fig. 5a). At 8, 16, and 24 weeks, Sr-Ti and Mg-Ti induced significantly higher failure load than cp-Ti and slightly, albeit insignificantly, higher failure load than Ca-Ti. Thus, Sr-Ti and Mg-Ti demonstrated better bone-bonding ability, especially at the early stage.

Fig. 5b shows the BIC results from the histological analysis. Mg-Ti at 4 weeks as well as Sr-Ti and Mg-Ti at 8 weeks showed higher BICs than Ca-Ti. This finding is consistent with the biomechanical testing results. At 16 and 24 weeks, the BICs were almost equal among the three treated sample types. As shown in Fig. 5c, at 4 weeks, all samples showed immature new bone formation near the implant. Notably, Sr-Ti and Mg-Ti highly promoted new bone formation and direct contact between implant and bone. At 8 weeks, bone formation along the implant occurred in the treated samples. New bone near the implant appeared to be maintained in the Sr-Ti- and Mg-Ti-treated groups. After 16 weeks, bone remodeling occurred in all samples, and the extra new bone was resorbed. While the bone near cp-Ti showed atrophic changes, Ca-Ti, Sr-Ti, and Mg-Ti equally maintained the near-implant bone volume.

The serum Sr ion concentration at 16 weeks was significantly higher than that in the preoperative period (Fig. 6a). In contrast, the serum Mg ion concentration did not change significantly (Fig. 6b). In bone element analysis of Sr at 4 weeks using XPS, Sr3s, attributed to a peak at approximately 359 eV, was detected at low levels in the bone near the implant (Fig. 6c). Inside the implant or at the posterior aspect of the tibia, Sr was not detected. At 8 weeks, similar results were obtained. Thus, the released Sr ions were taken up by the bone near the implant. As for Mg,

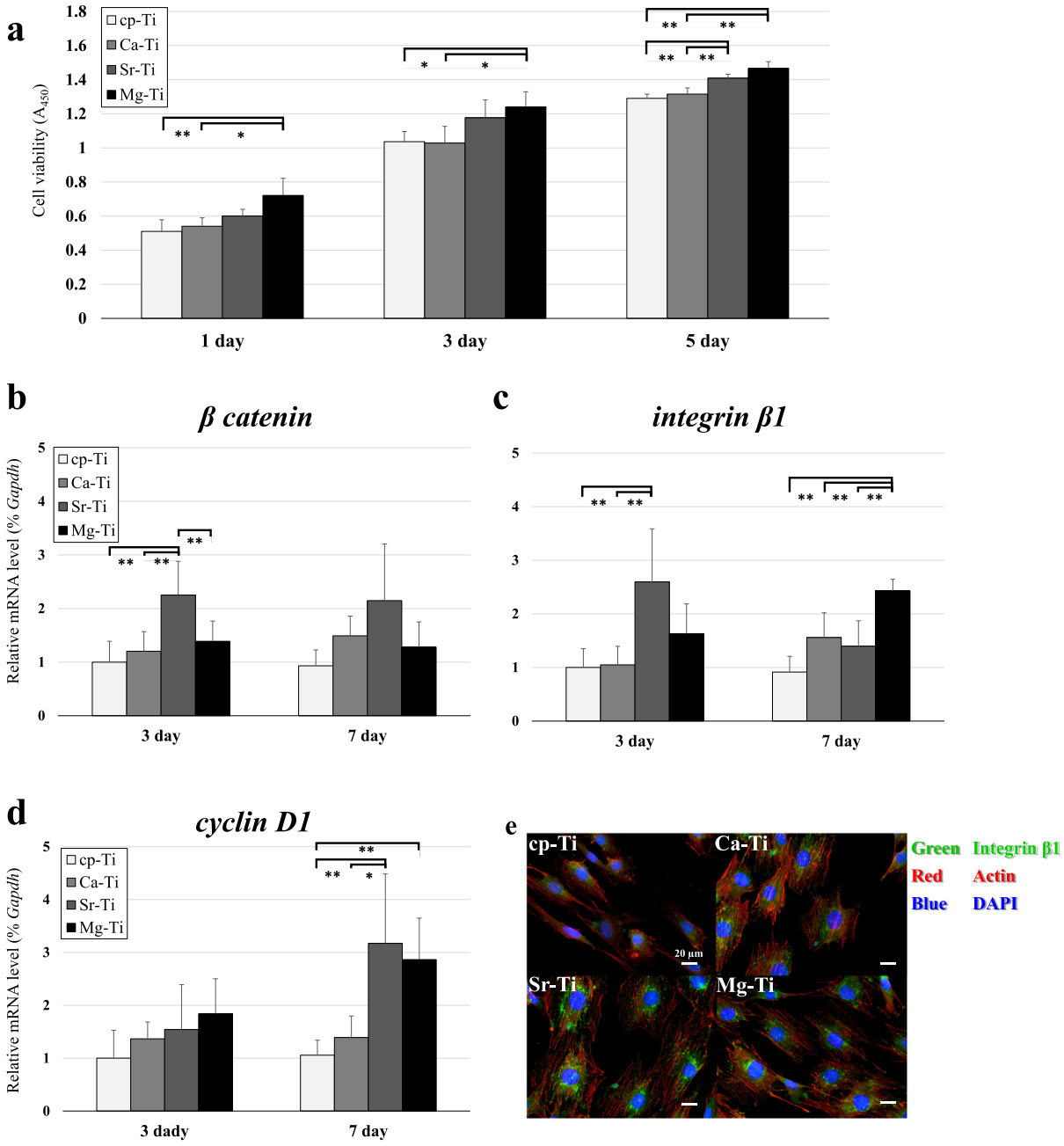

Fig. 2. Cell viability, gene expression of *β-catenin*, *integrin β1*, and *cyclin D1*, and immunofluorescence staining of integrin β1 of MC3T3-E1 cells cultured on each sample type. (a) Cytotoxicity of the three treated sample implant types as assessed by cell viability. ($n = 4$) (b–d) Relative gene expression of the indicated genes in the three treated samples and cp-Ti as measured by RT-qPCR. *Gapdh* was used as an internal control. ($n = 5$) (e) Cells on Sr- and Mg-Ti showed good cellular extension as well as higher expression and more extensive distribution of integrin β1 than those on Ca-Ti and cp-Ti. $^{*}p < 0.05$, $^{**}p < 0.01$.

Mg1s, attributed to a peak at approximately 1303 eV, was detected at both spots 1 and 2, and no apparent wave differences between these spots were observed (Fig. 6c).

4. Discussion

In the present study, the bioactivity of Ti releasing Sr or Mg ions was evaluated *in vitro* and *in vivo*. In the *in vitro* study, no cytotoxicity and higher osteogenic differentiation-related gene expression were observed for the three treated Ti samples, especially for Sr-Ti and Mg-Ti. Furthermore, ALP activity and ECM mineralization were higher for Sr-Ti and Mg-Ti than cp-Ti and Ca-Ti. In the *in vivo* study, greater failure load and new bone formation as well as higher BICs were observed in Sr-Ti and Mg-Ti at the early stage (4 and 8 weeks). In the long term (16 and 24 weeks), the failure load and BIC time-dependently increased, and near-implant bone volumes were maintained in animals that had received the three types of treated Ti. These results may be partially attributed to the higher expression of *β-catenin* in the Sr-Ti group, the higher expression of *cyclin D1* in the Sr- and Mg-Ti groups, and higher expression and broader distribution of integrin β1 in Sr-Ti and Mg-Ti than in Ca-Ti and cp-Ti groups.

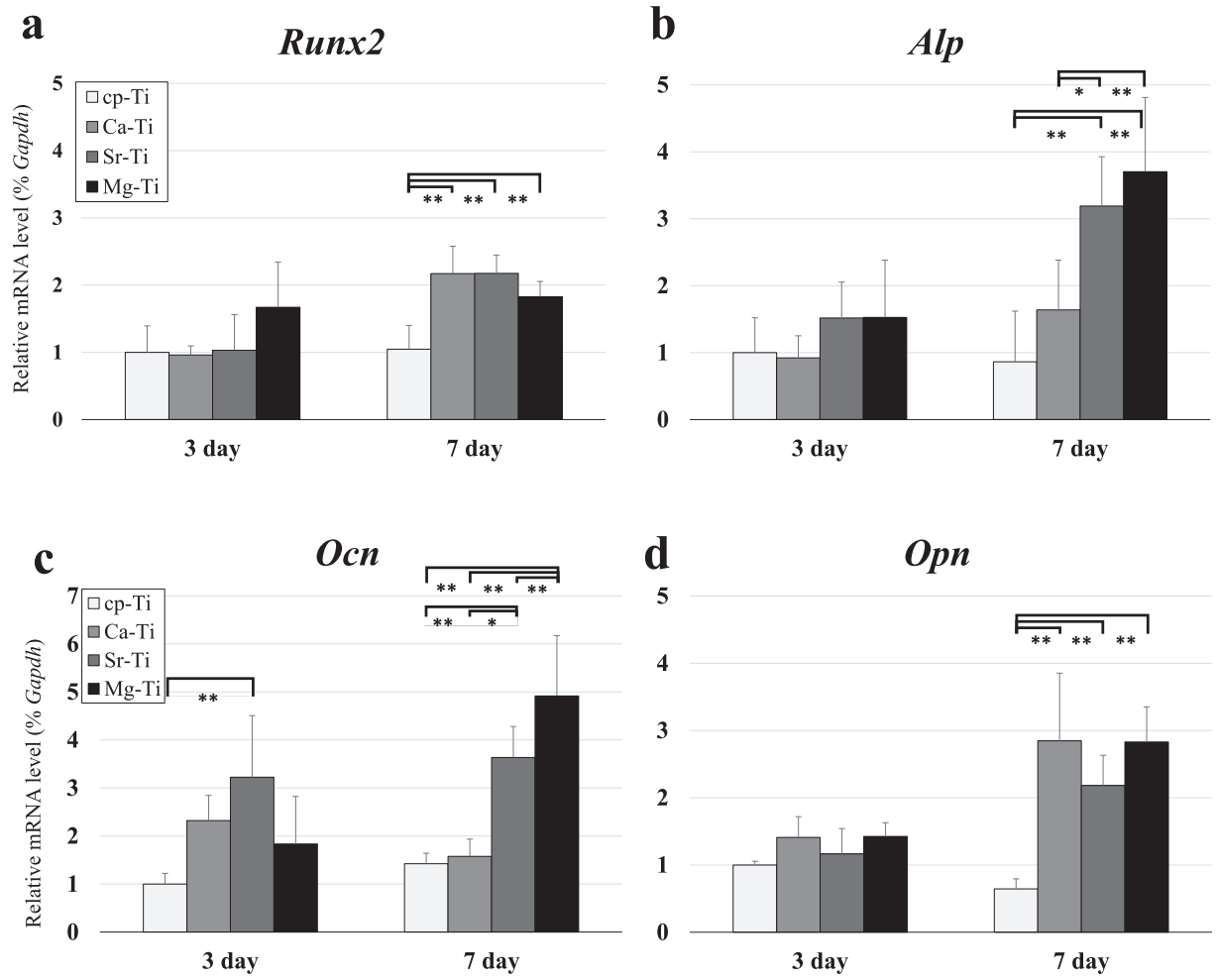

Fig. 3. Osteogenic gene expression of MC3T3-E1 cells cultured on each sample type. (a–d) Expression of the indicated osteogenic genes at different time points as measured by RT-qPCR. ($n = 5$), $^{*}p < 0.05$, $^{**}p < 0.01$.

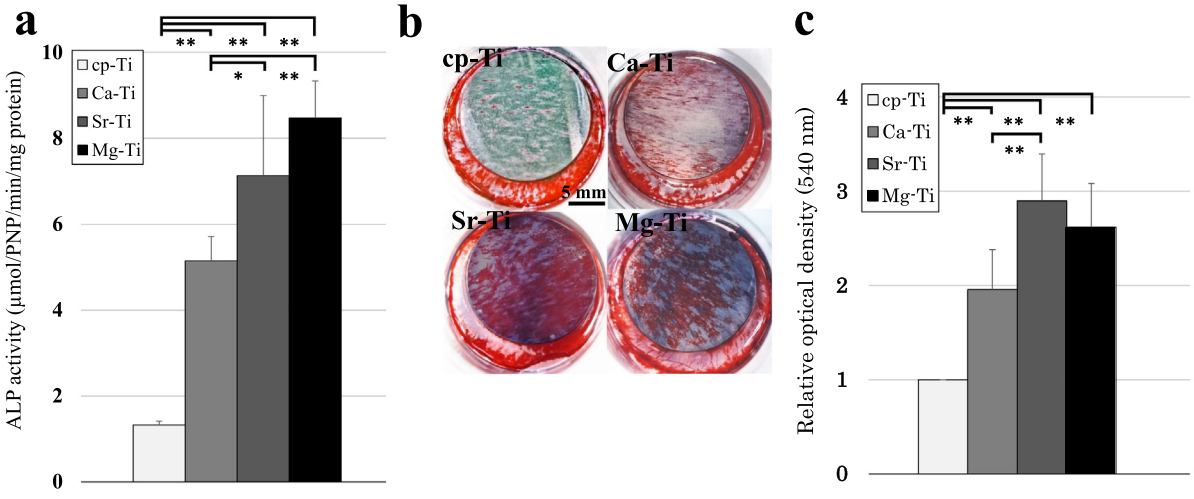

Fig. 4. ALP activity and ECM mineralization. (a) ALP activity of the treated samples compared to the untreated control (cp-Ti). (n = 6). (b) Representative images of Alizarin red staining of each sample type. (c) ECM mineralization of the treated samples as measured by Alizarin red staining. (n = 5), $^{*}p < 0.05$, $^{**}p < 0.01$.

Various types of trace elements, such as Sr, Mg, Zn, F, and Cu, are being investigated and utilized for bioactive implants [27]. To our knowledge, Sr is the most popular and most investigated, and has been shown to induce osteogenic differentiation [15]. In comparison, Mg is a relatively recent topic of bioactivity studies, and its associated cellular signals have not been established. In

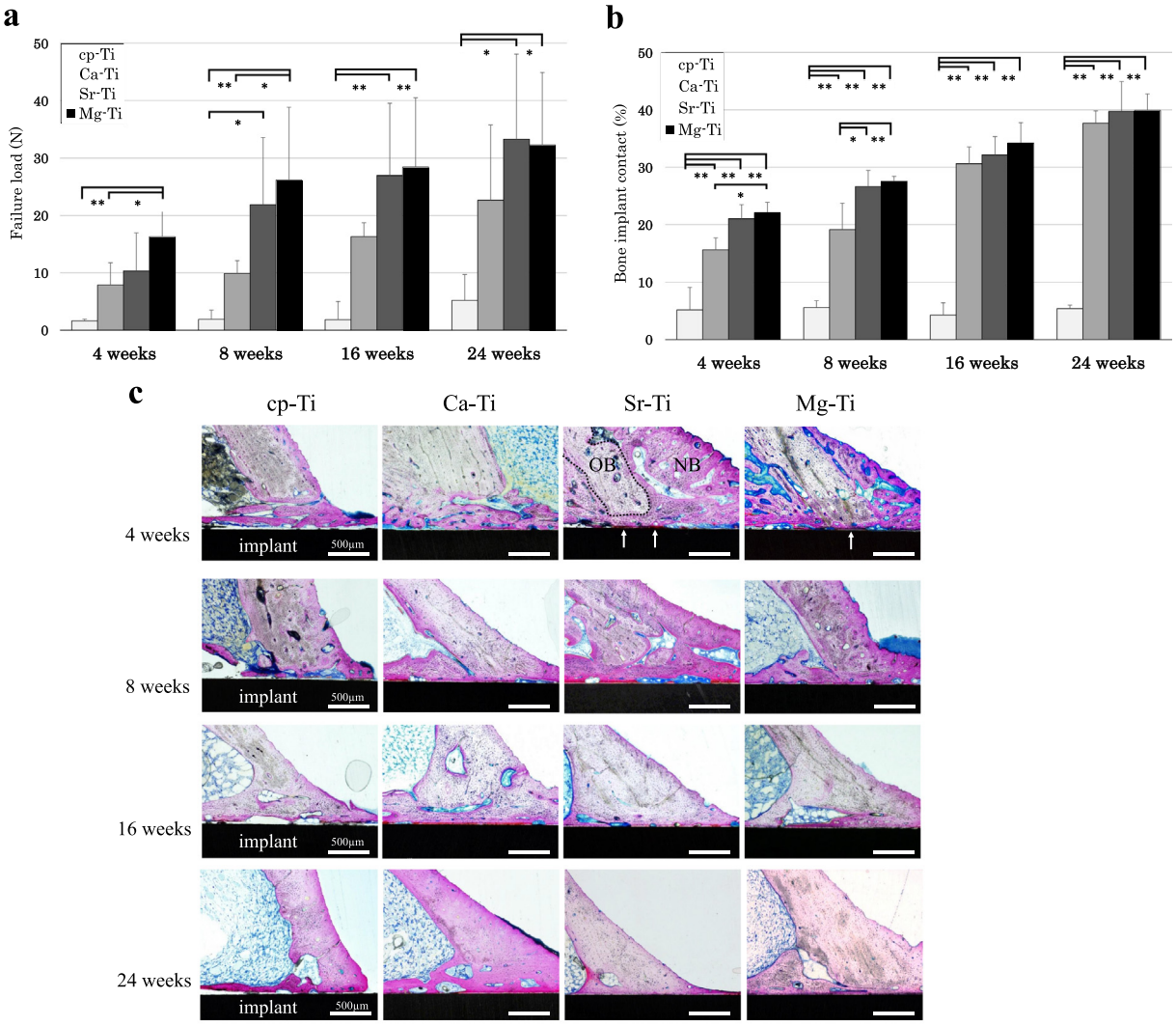

Fig. 5. Biomechanical test, histology, and histomorphometry. (a) Detachment test showing the bone-bonding strength of the samples. (cp-Ti; n = 3, Ca-, Sr-, and Mg-Ti; n = 6). (b) BIC ratios at different time points after implantation for the indicated samples. (pTi; n = 3, Ca-, Sr-, and Mg-Ti; n = 4). (c) New bone formation was observed at 4 weeks in all groups. Sr- and Mg-Ti showed direct bone contact between new bone and the sample surface (white arrows), although cp-Ti showed almost no contact with the bone. Although the extra new bone was resorbed owing to bone remodeling at 16 and 24 weeks, good contact between bone and samples was observed for the three treated sample types. NB, new bone; OB, old bone. $^{*}p < 0.05$, $^{**}p < 0.01$.

addition, implant studies using Sr or Mg have reported improved bioactivity both *in vitro* and *in vivo* [18,21,28]. However, in many of these studies, the implant treatments were complicated or required a special apparatus [21,28]. Conversely, our treatment methods are simple and of low cost, which is advantageous for clinical application. Another potential advantage is that our methods can be applied to a Ti surface regardless of its shape, because the process involves immersion and heating. Second, our methods only induce alterations on the scale of 2 μm at the surface of the implant and have almost no influence on implant composition and structure [9,23,24]. Third, the amount of ion release and surface composition of the element, especially Sr or Mg content, can be adjusted to some extent. For example, it has been shown that Sr and Mg contents in the surface layer can be varied from 0.6% to 2.8% and 0.3% to 2.6%, respectively, by increasing the Sr and Mg concentrations in the second treatment solution, whereas ion release was controlled from 0.06 to 0.92 ppm and from 0.02 to 0.43 ppm, respectively, by increasing the Sr and Mg concentrations in the final treatment solution [23,24]. Therefore, our methods are flexible, and thus likely to provide broad utility.

Notably, Sr-Ti and Mg-Ti showed higher bone-bonding ability than Ca-Ti, the positive control, at an early stage; this higher ability was maintained at a later stage, albeit not significantly. This early-stage superiority of Sr-Ti and Mg-Ti compared to that of Ca-Ti could be attributed to implant-derived Sr or Mg, because all three types of treated Ti had similar microporous surface structures [9,23,24]. In particular, the bone near Sr-Ti samples contained implant-derived Sr, suggesting that implant-derived Sr ions were released *in vivo* and could influence the tissue near the Sr-Ti. Accordingly, we concluded that Sr ions released from the implant impact osteoblasts or MSCs near the implant and enhance their osteogenic differentiation and activity, which allows earlier and more substantial new bone formation to occur, as shown in Fig. 5c. On the other hand, we could not determine implant-derived Mg level near the Mg-Ti samples, because Mg is naturally present in the bone to a certain extent, and the small amount of implant-derived Mg cannot be distinguished from naturally present Mg by XPS. We concluded that implant-derived Mg similarly affected the tissue near the implant as did Sr-Ti. Furthermore, we found that *β-catenin* expression was higher in Sr- and Mg-Ti

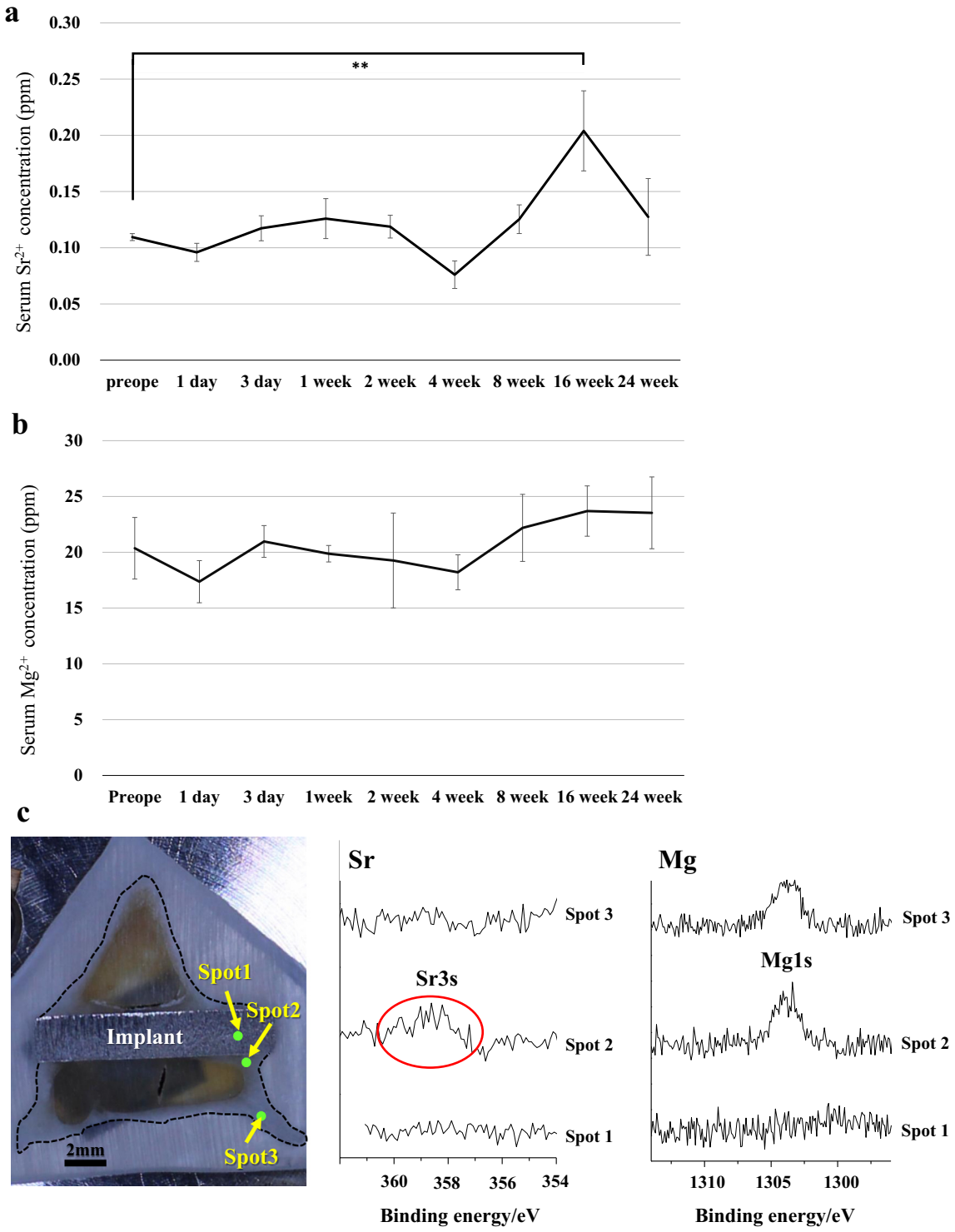

Fig. 6. Serum ion concentrations and bone element analysis. (a, b) The serum concentration of Sr ions was significantly elevated at 16 weeks, although no significant changes in Mg ion levels were observed. (n = 5). (c) Measurement locations (spots) on the thick section and results of XPS analysis. At spot 2, near the implant, Sr was detected at low levels at 4 weeks. At spots 1 and 3, Sr was not detected. Mg was detected at both spots 1 and 2, and no apparent wave differences were observed. Similar results were obtained both for Sr- and Mg-Ti at 8 weeks. $^{**}p < 0.01$.

(Fig. 4a). Beta-catenin is associated with transcription of various genes, and one of its most important targets during osteogenesis is *cyclin D1* [29], which encodes a key cell-proliferation factor. RT-qPCR results for *β-catenin* and *cyclin D1* at day 7 were comparable. In addition, the enhanced β-catenin activity suggests potential activation of the Wnt/β-catenin pathway, which is the main signaling mechanism utilized by Sr ions in promoting MSC proliferation and osteogenic differentiation [15,30].

Integrins binding to ligands induce various types of intracellular signaling, including the recruitment and activation of integrin-linked kinase (ILK), which leads to inhibition of glycogen synthase kinase-3β (GSK-3β) and inhibition of ubiquitin-mediated β-catenin

degradation; this consequently promotes nuclear translocation of β-catenin and osteogenic gene expression [31]. Integrin β1 reportedly plays an important role in osteoblast differentiation [32,33]. In our study, Sr- and Mg-Ti showed a wider distribution and higher expression of integrin β1 than Ca- and cp-Ti (Fig. 4), which might be one of the mechanisms underlying the earlier and enhanced new bone formation observed for these groups.

Several studies have reported the effect of osteogenic concentrations of Sr or Mg ions on MSCs or osteoblastic cells. Sila-Asna et al. [34] reported that Sr concentrations between 0.21 and 21.07 ppm in the cell culture medium increased ALP activity in human MSCs. Park et al. [35] reported that Sr ion release from $SrTiO_3$-coated implants was 0.75 ppm within 7 days and enhanced the attachment, spreading, focal adhesion, ALP activity, and expression of integrins and osteogenic genes in mouse BMSCs. Implanted Mg-releasing Ti provided increased spreading, proliferation, ALP activity, and gene expression of *Ocn* and *Opn* in rat BMSCs, with a released Mg ion concentration of 0.5–1 ppm [36]. These ion concentrations are similar to the results of our previous study, which showed that Sr or Mg ion release from Ti was 0.92 or 0.43 ppm, respectively, over 7 days. However, other studies have reported much higher concentrations; 0.1–5 mM has been reported in studies using strontium ranelate [37–39] and 5–10 mM in studies using Mg ions [19,20,40]. These discrepancies may arise for several reasons. First, the surface-contained Sr or Mg as well as the released ions may influence the cellular response. In addition, the ion concentration immediately adjacent to the implant surface might be much higher than the measured concentration. Finally, the study methods in these reports differ fundamentally. Most studies reporting lower ion concentrations comprise implant-related studies describing higher "bioactivity," while higher concentrations are generally reported in cell culture studies, where the Sr or Mg ions were added to the culture medium to induce a cellular "osteogenic response".

In our study, serum Sr concentrations were significantly higher at 16 weeks postoperatively, which was rather unexpected. We cannot completely rule out the possibility of some issues with collection, preservation, and measurement of the blood samples. However, the increase was only 0.1 ppm and the maximum concentration was only 0.2 ppm; thus, the concentrations remained at a less-than-toxic level over the postoperative periods. The serum Sr concentration in humans reportedly varies widely between approximately 10 μg/l and 100 mg/l [41–43] due to regional differences in the Sr content of soil and water. Furthermore, the serum concentration of Sr after ingestion of 2.0 g of strontium ranelate is approximately 8 mg/l at maximum, with a baseline concentration of 5 μg/l [44]. Therefore, despite the specific differences between rabbits and humans, an increase of only 0.1 ppm and the maximum concentration of only 0.2 ppm are not likely to be relevant in humans. With respect to porous Sr-Ti, which has a much larger surface area than the smooth surface used in the present study, careful monitoring of serum concentrations will be required. This issue will need to be addressed in future studies.

The present study had some limitations. First, the mechanism underlying the bioactivity provided by this method is not fully elucidated, although we provided evidence supporting the possible mechanisms of the Wnt/β-catenin signaling and cell adhesion related to integrin β1 *in vitro*. Second, we used MC3T3-E1 cells in the *in vitro* study and rabbits in the *in vivo* study. Owing to the recognized differences between model animals and humans, the possible mechanism and bioactivity of this method cannot be generalized to humans, although the present preliminary investigation of the mechanism, safety, and effectiveness of the materials in animals is essential. Third, it is difficult to evaluate the influence of surface topography, as the treatments affected not only the topography but also the chemical composition of the surface. Fourth, the effectiveness of our technique for Ti alloys, which are more widely used for clinical applications than pure Ti, was not determined. Fifth, we have not investigated the combination of Sr and Mg. A synergistic effect of these two ions may occur, as some studies have reported that the combination of different ions produces such effects [45,46]. Further studies are needed to resolve these limitations.

5. Conclusions

Surface treatment of Ti to produce Ti that releases Sr or Mg ions resulted in higher cell viability and promoted osteoblast differentiation in MC3T3-E1 cells. Furthermore, an *in vivo* study using rabbits demonstrated that Sr-Ti and Mg-Ti implants showed higher bioactivity, especially at the early stage, and that the bone-bonding strength and BIC were maintained in the long term. The main advantages of this method are its simplicity, applicability to any shape of Ti, and lack of adverse effects on the composition, shape, and mechanical strength of the Ti materials. Therefore, our method is a promising treatment for achieving early bone bonding of orthopedic or dental Ti-based implants in clinical applications. Further studies are needed to elucidate the mechanism underlying the bioactivity provided by this treatment.

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