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ORIGINAL ARTICLE

TERM

Extracellular Calcium-Binding Peptide-Modified Ceramics Stimulate Regeneration of Calvarial Bone Defects

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Secreted protein, acidic, cysteine-rich (SPARC)-related modular calcium binding 1 (SMOC1) has been implicated in the regulation of osteogenic differentiation of human bone marrow mesenchymal stem cells (BMSCs). In this study, we found that a peptide (16 amino acids in length), which is located in the extracellular calcium (EC) binding domain of SMOC1, stimulated osteogenic differentiation of human BMSCs *in vitro* and calvarial bone regeneration *in vivo*. Treatment of BMSCs with SMOC1-EC peptide significantly stimulated their mineralization in a dose-dependent manner without changing their rate of proliferation. The expression of osteogenic differentiation marker genes, including type 1 collagen and osteocalcin, also increased in a dose-dependent manner. To examine the effect of the SMOC1-EC peptide on bone formation *in vivo*, the peptide was covalently immobilized onto hydroxyapatite/β-tricalcium phosphate (HA/β-TCP) particles. X-ray photoelectron spectroscopy analysis showed that the peptide was successfully immobilized onto the surface of HA/β-TCP. Implantation of the SMOC1-EC peptide-immobilized HA/β-TCP particles into mouse calvarial defects and subsequent analyses using micro-computed tomography and histology showed significant bone regeneration compared with that of calvarial defects implanted with unmodified HA/β-TCP particles. Collectively, our data suggest that a peptide derived from the EC domain of SMOC1 induces osteogenic differentiation of human BMSCs *in vitro* and efficiently enhances bone regeneration *in vivo*.

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Key Words: Secreted protein, acidic, cysteine-rich-related modular calcium binding 1; Extracellular calcium domain; Peptide; Hydroxyapatite/β-tricalcium phosphate; Osteogenesis; bone marrow mesenchymal stem cells

INTRODUCTION

Bioactive peptides are defined as particular protein fragments derived from either natural or artificial proteins [1]. They have been implicated in regulating a variety of biological functions to exert antioxidant [2], antimicrobial [3], antihypertensive [4], antithrombotic [5], and immunomodulatory activities [6]. Bioactive peptides can also mimic the biological activities of proteinaceous growth factors. In particular, peptides derived from bone morphogenetic proteins (BMPs) have activities similar to those of full-length BMPs and can stimulate bone formation [7]. Although BMPs have been applied for the therapeutic treatment of bone defects or diseases [8], their clinical application requires careful consideration because they may cause severe adverse effects such as organogenesis, apoptosis, tumorigenicity, and immunogenicity [9,10]. In addition, the application of concentrated BMPs without immobilization occasionally leads to ectopic bone formation [10], and their long-term effects and release patterns are still unknown. To overcome these issues, the controlled release of BMPs after their conjugation to materials is necessary. In the conjugation process, however, peptides have the advantage of better stability compared with that of native proteins. Moreover, peptides can be massively and economically synthesized in laboratories. Biomaterials modified with growth factor-derived biomimetic peptides can stimulate cellular healing or regeneration, and thus can be used to enhance the performance of scaffolds for hard tissue engineering [11].

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For the regeneration of hard tissue, a variety of biomaterials has been developed, including bioactive glasses, ceramics, and other composites. In particular, hydroxyapatite-based ceramics have been widely used for orthopedic and dental surgeries because they have osteoconductive properties [12]. Recently, hydroxyapatite/β-tricalcium phosphate (HA/β-TCP) composite ceramics have been broadly used for the regeneration of bone defects because of their degradability, biocompatibility, and osteoconductivity [13]. However, their biological performance needs to be improved to enhance bone regeneration. An approach that may achieve such an improvement in performance is the immobilization of bioactive molecules onto the HA/β-TCP composite ceramics. Previous reports have shown that surface modification of ceramics with bioactive peptides resulted in enhanced bone regeneration [14,15].

Previously, we found that SPARC-related modular calcium binding 1 (SMOC1) plays an important role in regulating the osteogenic differentiation of human bone marrow mesenchymal stem cells (BMSCs) [16]. SMOC1 was first identified as a member of the SPARC (BM-40 or osteonectin) family and is a calcium-binding protein in the basement membrane [17]. It binds to several extracellular matrix (ECM) proteins and plays roles in bone remodeling and cancer metastasis [18]. SMOC1 was reported to be downregulated by cytokines and nitric oxide in rat mesangial cells [19]. On the other hand, SMOC1 mutations in humans are known to cause a genetic disorder called the Waardenburg Anophthalmia Syndrome, which results in abnormalities of the limbs and eyes [20]. SMOC1 has distinct domains, including 1 follistatin-like domain, 2 thyroglobulin-like domains, 1 extracellular calcium (EC) domain, and 1 SMOC1 unique domain [21]. The EC domain has calcium-binding sites, and calcium ions are critical for osteogenic differentiation and mineralization. We selected peptide sequences from each domain of SMOC1, and we found that a peptide fragment derived from the EC domain of SMOC1 stimulated osteogenic differentiation of human BMSCs *in vitro* and bone regeneration *in vivo*.

MATERIALS AND METHODS

Materials

TriOSite™ was purchased from Zimmer (Vigneux-de-Bretagne, France) to generate SMOC1-EC peptide-immobilized HA/β-TCP particles. For peptide immobilization, HA/β-TCP granules were ground with a mortar, and sieved to a size of 100–200 μm. Poly (ethylene glycol disuccinimidyl succinate) [PEG-$(SS)_2$] and 3-aminopropyl-triethoxysilane (APTES) were obtained from SunBio (Anyang, Korea) and Sigma-Aldrich (Milwaukee, WI, USA), respectively. The amino acid sequence of the SMOC1-EC peptide was selected from the region of the second α-helix of the EF-hand calcium-binding motif within the EC domain. The SMOC1-EC peptide was optimized for net charge and solubility using Peptide Property Calculator (http://www.basic.northwestern.edu/biotools/proteincalc.html). The SMOC1-EC peptide consists of 16 amino acids covering residues 399–414 in the human SMOC1 peptide sequence. The isoelectric point of the peptide was 8.470 and its average hydrophilicity was 0.92. The percentage of hydrophilic residues among total residues was 56.25%. The molecular weight of the peptide was 1976.45 Da, and its net charge at pH 7.0 was 1.9. The SMOC1-EC peptide (KCARRFTDYCDLNKDK) was synthesized with 95% purity by Peptron (Daejeon, Korea).

Immobilization of the SMOC1-EC peptide onto the surface of HA/β-TCP particles

The surface immobilization procedure was described previously [14]. SMOC1-EC peptide was immobilized to HA/β-TCP particles (100–200 μm) using 3 steps. First, the free hydroxyl groups of the HA/β-TCP particles were silanized by treatment with the primary amine APTES (500 mM) in anhydrous ethanol for 6 h at 25°C. Second, the silanized HA/β-TCP particles were washed with ethanol 5 times and then reacted with 10 mM of PEG-$(SS)_2$ in 2:3 dimethylformamide and ethanol solution for another 6 h. Third, the PEGylated particles were rinsed with ethanol and phosphate buffered saline and then reacted with 10 μM of SMOC1-EC peptide with gentle rotation for 12 h at 4°C. The processed HA/β-TCP was subsequently cleaned with ethanol and freeze-dried before storage.

Validation of the immobilization of SMOC1-EC peptide onto HA/β-TCP

Dried PEGlyated and SMOC1-EC peptide-immobilized HA/β-TCP particles were analyzed using X-ray photoelectron spectroscopy (XPS) (Quantera SXM, ULVAC-PHI, Japan). The XPS spectra were obtained using algorithms of a magnesium anode at 13 kV and 30 mA. Further analyses of XPS and XPS-C1s were followed as described earlier [14]. The XPS-C1s band spectra were separated into multi-peak spectra using ORIGIN 6.0 software (OriginLab Corp., Northampton, MA, USA).

Osteogenic differentiation and cytotoxicity of human BMSCs treated with SMOC1-EC peptide

To examine the effect of the synthetic SMOC1-EC peptide on osteogenic differentiation *in vitro*, human BMSCs were seeded at a density of 1.3×10^4 cells per cm^2 on a culture plate (Falcon, San Jose, CA, USA), in triplicate. After 24 h, they were treated with osteogenic induction medium [α-MEM with 10% (v/v) fetal bovine serum, 1% (v/v) antibiotics solution, 50 μg/mL ascorbic acid, 10 nM dexamethasone, and 10 mM β-glycerophos-

phate] containing SMOC1-EC peptides (0, 0.1, 0.5, or 2.5 μM). After 21 days, the cells were stained with Alizarin red S. The mineral depositions were visualized with a light microscope (Olympus, Tokyo, Japan), and dye extracted using 10% (w/v) cetylpyridinium chloride was measured at OD_{570} as described previously [22]. To analyze the effect of the SMOC1-EC peptide on the expression of osteogenic differentiation marker genes, total ribonucleic acid was isolated using TRI-Solution™ (Bio Science Korea, Gyeongsan, Korea), and complementary DNA was synthesized using SuperScript™ II (Invitrogen, Carlsbad, CA, USA). The subsequent thermal cycling was analyzed using a C1000™ thermal cycler (Bio-Rad, Foster City, CA, USA). The osteogenic differentiation marker genes included osteonectin (*ON*), osteocalcin (*OC*), and type 1 collagen (*COL1*). The primer sequences of *ON*, *OC*, and *COL1* were described previously [16]. Each sample was analyzed in triplicate and normalized to *GAPDH* expression. To examine the cytotoxicity of the SMOC1-EC peptide, 700 human BMSCs were seeded in each well of a 96-well plate. The cells were treated with varying concentrations (0, 0.1, 0.5, and 2.5 μM) of SMOC1-EC peptide, and cytotoxicity assessment was performed using the methylthiazol tetrazolium (MTT) assay (Sigma-Aldrich) as described previously [23].

Animal study

Animal experiments were performed under the guidance of the Institutional Ethics Committee of Kyungpook National University. Healthy Institute for Cancer Research (ICR) mice (male, 6 weeks old) were used for the experiment, and anesthetization was performed as previously described [24]. The head was shaved and cleaned up with povidone-iodine solution and the parietal skin and periosteum were incised. The calvarial defect was generated by trephine burr (4 mm in diameter) with a dental hand piece (Marathon, Seoul, Korea). SMOC1-EC peptide-immobilized HA/β-TCP particles (10 mg) were mixed with fibrin glue (Greenplast kit VR, Green Cross, Seoul, Korea) [25] and molded into a flattened disc of 4 mm diameter and 1 mm thickness. Then, the disc-shaped particles were implanted into the defect region of the calvariae. Animals were implanted with either unmodified HA/β-TCP (n=5) or SMOC1-EC-immobilized HA/β-TCP (n=5). Six weeks after implantation, the mice were sacrificed and the calvariae were isolated and fixed with 4% (w/v) formaldehyde in phosphate buffered saline (pH 7.4) for 48 h. The specimens were decalcified with 0.5 M ethylenediaminetetraacetic acid (pH 8.0) for 7 days and then processed for paraffin embedding. The paraffin blocks were sectioned at a thickness of 5 μm using a microtome (Leica Microsystems, Nussloch, Germany), and sections were stained with hematoxylin and eosin (H&E) and Masson's trichrome. The new bone area was measured and analyzed using IMT i-Solution software (IMT Technology, Daejeon, Korea).

Analysis of micro-computed tomography

To analyze the regeneration of the calvarial defects, the fixed calvariae were scanned using an X-eye micro-CT system (SEC, Seoul, Korea) with an anode current of 80 μA and an anode voltage of 72 kV. The imaging data were achieved using half-scan CT algorithms, and scanned planes of 512×512 were reconstructed using the HARMONY program (DRGEM, Seoul, Korea). Subsequently, 3-dimensional (3D) images were created using the iCAT3D program (Mevisys, Daejeon, Korea). Reconstructed 3D images were further analyzed using CTAn (Bruker microCT, Kontich, Belgium) to calculate the bone volume as a proportion of tissue volume (%).

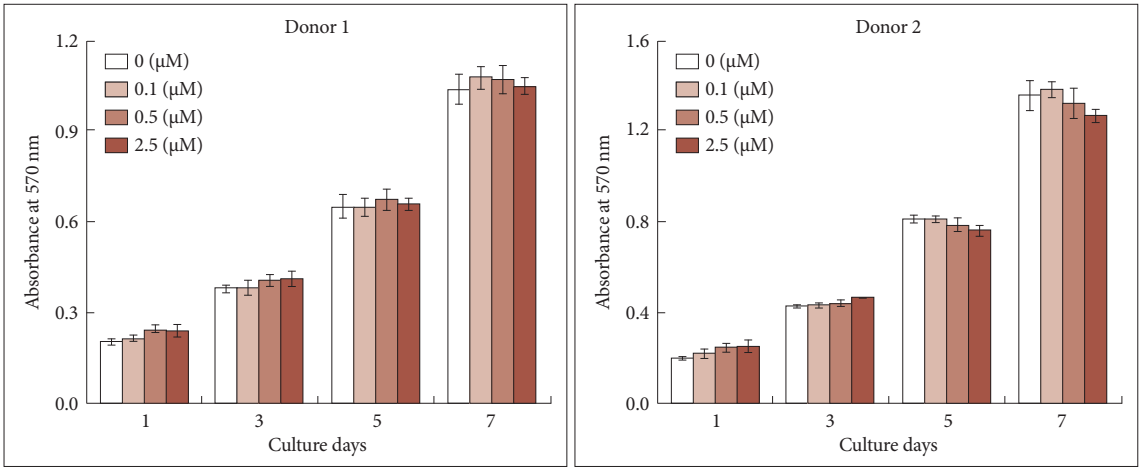


Figure 1. Cytotoxicity of SMOC1-EC peptide in human BMSCs. Human BMSCs were seeded in 96 well plates and treated with increasing doses of SMOC1-EC peptides for 1 week. At the indicated time point, MTT assay was performed (n=3). There are no statistical differences. SMOC1-EC: SPARC-related modular calcium binding 1-extracellular calcium, BMSCs: bone marrow mesenchymal stem cells, MTT: methylthiazol tetrazolium.

Statistical analysis

Statistical analysis was performed with SPSS 21.0 (one-way analysis of variance) and *p* values of less than 0.05, 0.02, or 0.01 were considered significant.

RESULTS

Cytotoxicity of SMOC1-EC peptide in human BMSCs

The cytotoxicity of the SMOC1-EC peptide was examined using the MTT assay. Human BMSCs isolated from 2 independent donors were treated with SMOC1-EC peptide at the concentrations of 0, 0.1, 0.5, and 2.5 μM, and MTT assay was performed after 1, 3, 5, and 7 days of culture. The results showed that the SMOC1-EC peptide did not significantly affect the proliferation of BMSCs derived from donor 1 or donor 2, compared with the control (Fig. 1). These results suggest that the SMOC1-EC peptide did not significantly exert cytotoxicity in human BMSCs.

Effect of SMOC1-EC peptide on mineralization and osteogenic differentiation of human BMSCs

To examine the effect of the SMOC1-EC peptide on the osteogenic differentiation of BMSCs, human BMSCs were cultured in osteogenic induction media with or without the indicated concentrations of the SMOC1-EC peptide for 3 weeks and mineralization was evaluated by Alizarin red S staining. The results showed that the SMOC1-EC peptide significantly increased calcium deposition in human BMSCs in a concentration-dependent manner (Fig. 2A, left). Quantification of the extracted dye confirmed an increase in calcium deposition by the SMOC1-EC peptide compared with the control ($p<0.01$) (Fig. 2A, right). These results suggest that the SMOC1-EC peptide stimulated osteogenic differentiation and mineralization in human BMSCs.

To further evaluate whether osteogenic differentiation of human BMSCs is enhanced by the SMOC1-EC peptide, human BMSCs were stimulated with or without SMOC1-EC peptide in osteogenic induction media for 2 weeks and the messenger ri-

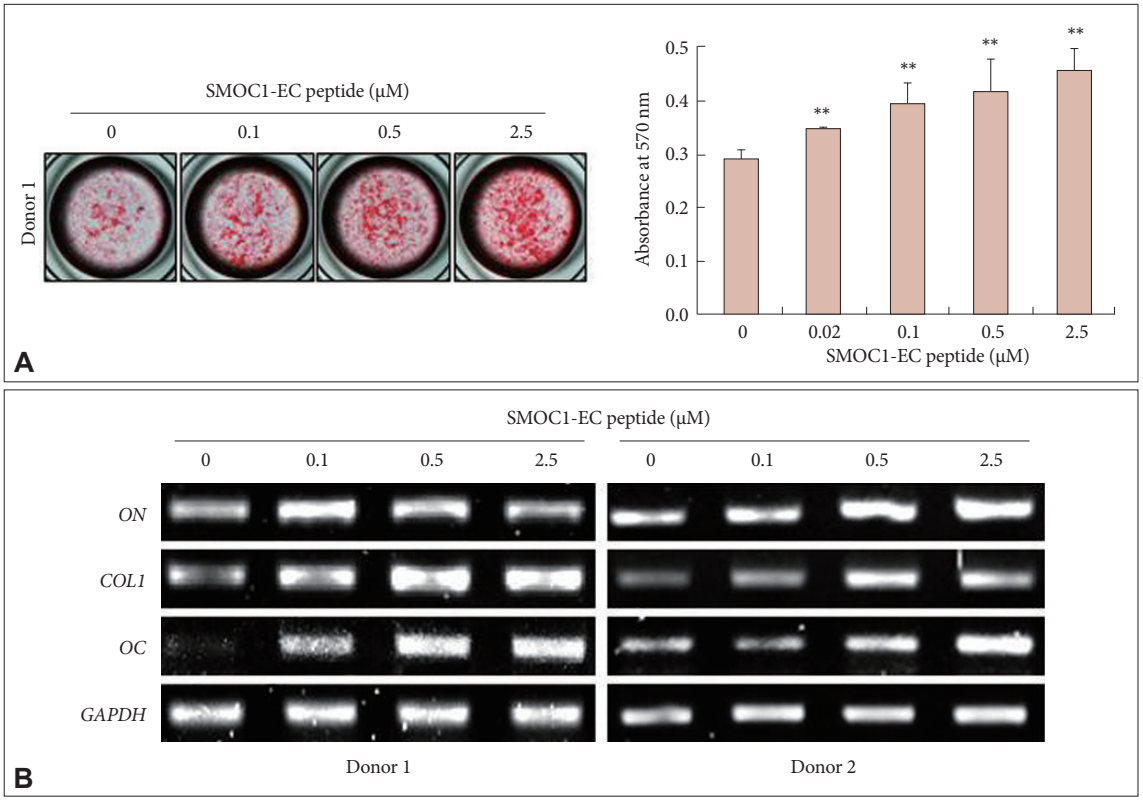
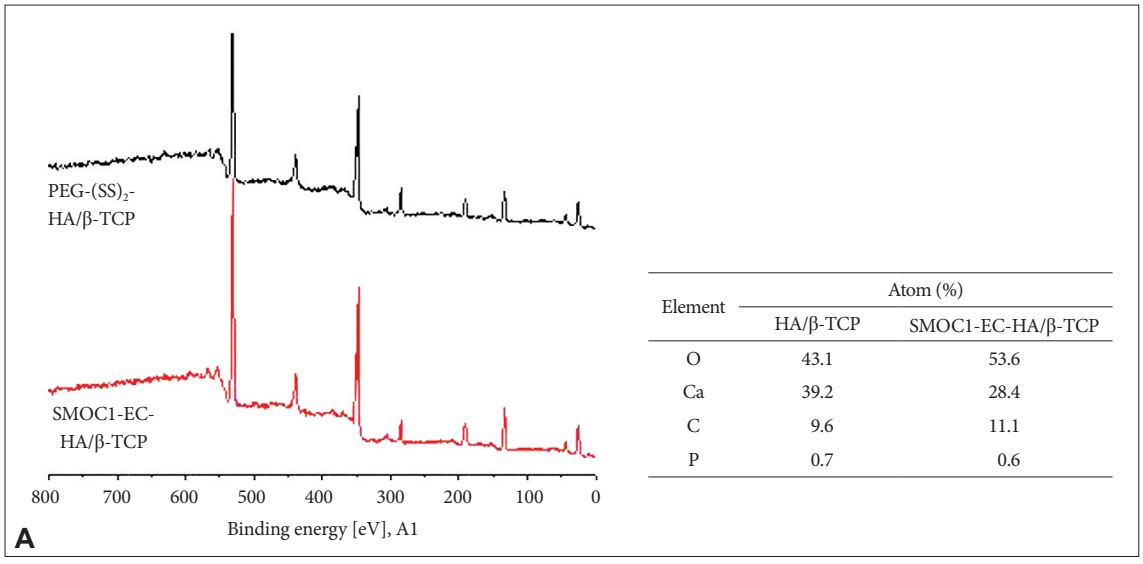


Figure 2. *In vitro* effect of the SMOC1-EC peptide on osteogenic differentiation and mineralization of human BMSCs. (A) Osteogenic differentiation of human BMSCs was induced with osteogenic induction medium and indicated concentrations of SMOC1-EC peptides for 3 weeks, and subsequently stained with Alizarin red S (left). The stained Alizarin red S dye was extracted using 10% cetylpyridinium chloride and absorbance was measured at 570 nm (right) $^{**}p<0.01$. (B) Expression of osteoblast differentiation marker genes was evaluated by RT-PCR. Human BMSCs were induced with osteogenic induction medium with or without SMOC1-EC peptides for 2 weeks, and the expression levels of *ON*, *COLI*, and *OC* were determined by RT-PCR. *ON*: osteonectin, *COL1*: collagen type 1, SMOC1-EC: extracellular calcium-binding domain of SPARC-related modular calcium binding 1, *OC*: osteocalcin, BMSCs: bone marrow mesenchymal stem cells, RT-PCR: reverse transcriptase-polymer chain reaction.

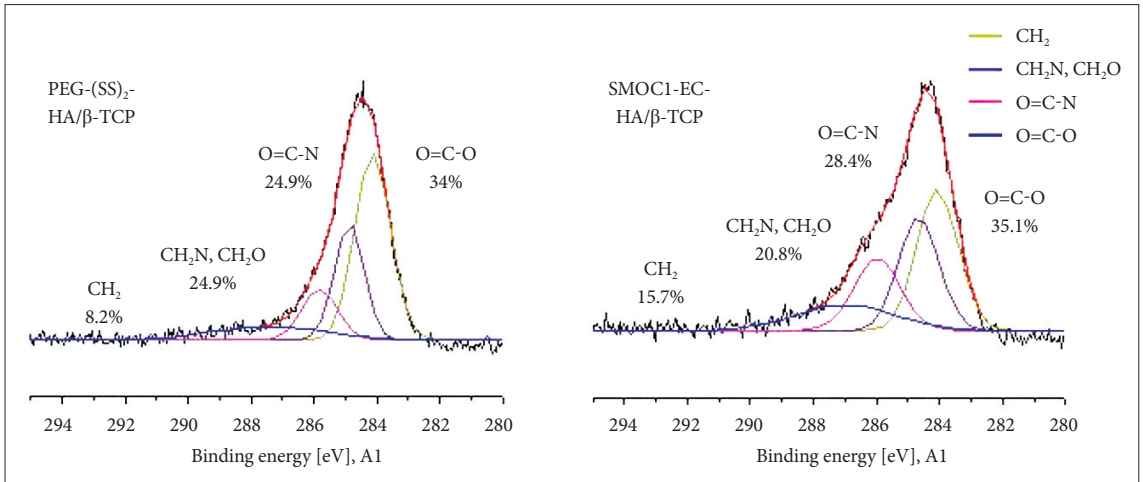
bonucleic acid expression of osteogenic differentiation markers was measured using reverse transcriptase-polymer chain reaction (RT-PCR). Conventional RT-PCR analysis showed that the expression of *ON*, *COL1*, and *OC* was dose-dependently increased by SMOC1-EC peptide in BMSCs isolated from donor 1 (Fig. 2B). Similar induction of the expression of marker genes by SMOC1-EC peptide was observed in BMSCs isolated from donor 2 (Fig. 2B). However, the expression of *ALP*, an early osteoblast differentiation marker gene, was not significantly induced by the SMOC1-EC peptide (data not shown). These results suggest that the SMOC1-EC peptide stimulates the osteogenic differentiation of human BMSCs through inducing the expression of osteogenic differentiation marker genes that are possibly involved in the late stage of differentiation.

Surface analysis of the HA/β-TCP particles immobilized with SMOC1-EC peptide

Since the synthesized SMOC1-EC peptides induced osteogenic differentiation and mineralization of human BMSCs, we asked whether treatment with HA/β-TCP particles with SMOC1-EC peptide chemically immobilized onto their surfaces could improve bone regeneration *in vivo*. The SMOC1-EC pep-



Element	Atom (%)	
	HA/β-TCP	SMOC1-EC-HA/β-TCP
O	43.1	53.6
Ca	39.2	28.4
C	9.6	11.1
P	0.7	0.6



Groups	C=O/C-H ratio (%)
PEG-$(SS)_2$-HA/β-TCP	27.6
SMOC1-EC-HA/β-TCP	59.3

Figure 3. XPS analysis. (A) XPS spectra of PEG-$(SS)_2$-immobilized HA/β-TCP and SMOC1-EC peptide-immobilized HA/β-TCP. (B) XPS-C1s spectra of SMOC1-EC peptide-immobilized HA/β-TCP and PEG-$(SS)_2$-immobilized HA/β-TCP. XPS: X-ray photoelectron spectroscopy, PEG-$(SS)_2$: poly (ethylene glycol disuccinimidyl succinate), HA/β-TCP: hydroxyapatite/β-tricalcium phosphate, SMOC1-EC: SPARC-related modular calcium binding 1-extracellular calcium.

tides were immobilized onto HA/β-TCP as described in the Materials and Methods, and the efficiency of immobilization was examined with XPS analysis. The results showed that the total atomic percentage of carbon increased from 9.6% to 11.1% (Fig. 3A). Moreover, the carbon peaks of the SMOC1-EC peptide-immobilized HA/β-TCP increased in the form of CH_2 from 34% to 35.1% and in the carboxyl carbon O=C-O from 8.2% to 15.7% in comparison with unmodified HA/β-TCP (Fig. 3B). The ratio of C=O to C-H was increased by 31.7% in the SMOC1-EC peptide-immobilized HA/β-TCP (Fig. 3B). These results clearly suggest that the SMOC1-EC peptide was covalently immobilized onto the surface of HA/β-TCP.

In vivo calvarial bone regeneration by SMOC1-EC peptide-immobilized HA/β-TCP

To analyze the effect of the SMOC1-EC peptide-immobilized HA/β-TCP particles on bone regeneration *in vivo*, SMOC1-EC peptide-modified or unmodified HA/β-TCP particles were implanted into the calvarial defect area of mice as described in the Materials and Methods. After 6 weeks, the mice were sacrificed and the implanted calvariae did not show clinical signs of inflammation or adverse effects. Micro-CT images were obtained as top (periosteum side) and inside (cerebral side) views (Fig. 4A). The calvarial defect area implanted with HA/β-TCP was still partially separated from the host bone, whereas the defect area implanted with SMOC1-EC peptide immobilized HA/β-TCP was well covered (Fig. 4A), implying substantial bone regeneration in the mice treated with SMOC1-EC peptide immobilized HA/β-TCP. Analysis of the regenerated bone volume as a proportion of tissue volume in the defected area demonstrated that the ratio of the mineralized tissue volume to the defected tissue volume was increased by about 10% in the SMOC1-EC peptide-immobilized HA/β-TCP group compared with the unmodified HA/β-TCP group ($p<0.05$) (Fig. 4B). To examine the characteristics of newly formed bone, histomorphometric analysis was conducted (Fig. 5). The calvariae were decalcified, sectioned, and stained with H&E. The results showed that unmodified HA/β-TCP was surrounded by fibrous tissue, indicating that new bone did not form properly (Fig. 5A and C). A few initiation centers of bone regeneration were seen in the calvariae implanted with unmodified HA/β-TCP (Fig. 5C, open arrows). On the other hand, the implantation of SMOC1-EC peptide-immobilized HA/β-TCP was found to have produced substantial bone regeneration (Fig. 5B). Newly formed bone was connected with the host bone (Fig. 5B, black arrow). The newly formed bone tissue contained mature bone (lamination and lacunae) (Fig. 5D, black arrowhead) as well as woven bone (Fig. 5D, yellow arrowhead). Moreover, well-organized bone-lining cells (green arrowhead) and blood vessels (blue arrowhead) were observed

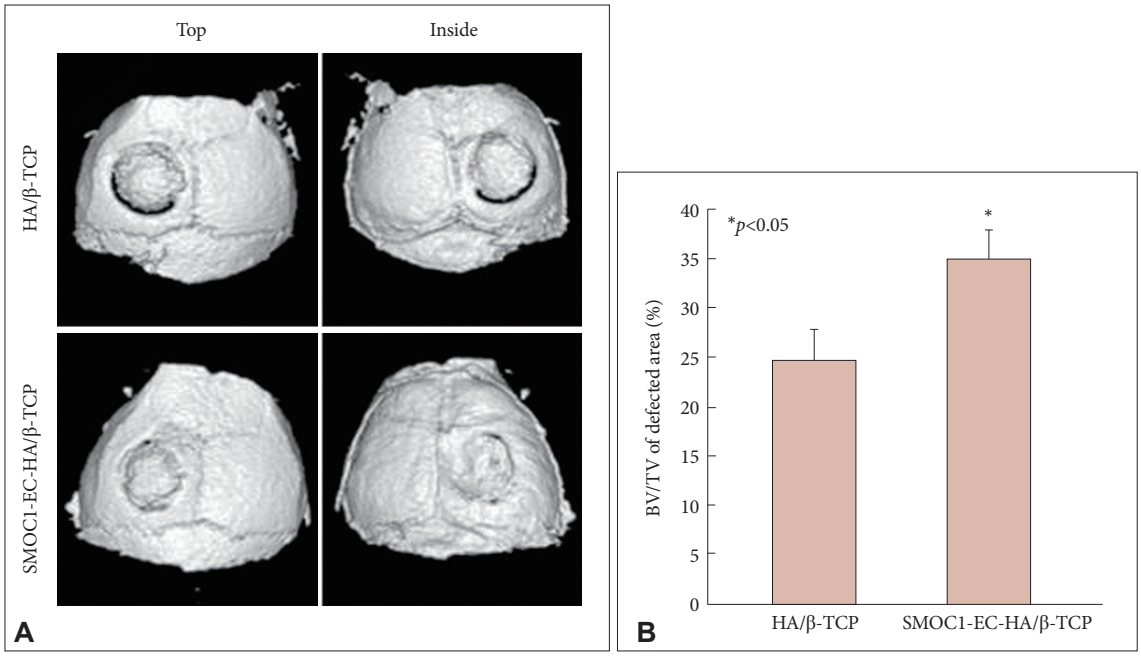


Figure 4. Micro-CT analysis of mouse calvariae with a defected area. (A) Six weeks after implantation, calvariae were scanned using micro-CT and 3D images were obtained. Upper panels are calvariae implanted with unmodified HA/β-TCP and lower panels are calvariae implanted with SMOC1-EC peptide-immobilized HA/β-TCP. The top (periosteum) and inside (cerebral) views of each calvaria are shown. (B) The ratio of bone volume to tissue volume in the defected area was quantified by CTAn program using 3D images. 3D: 3-dimensional, HA/β-TCP: hydroxyapatite/β-tricalcium phosphate, SMOC1-EC: SPARC-related modular calcium binding 1-extracellular calcium.

(Fig. 5D). The ratio of bone to tissue volume in the calvariae implanted with SMOC1-EC peptide-immobilized HA/β-TCP was 30.4% higher (p<0.02) than in those implanted with unmodified HA/β-TCP (Fig. 5F). To evaluate the maturation of newly formed bone further, Masson's trichrome staining was conducted. The implanted area around unmodified HA/β-TCP was stained with blue color, indicating that collagen matrix was well accumulated (Fig. 5E). In contrast, tissue areas where SMOC1-EC peptide-immobilized HA/β-TCP particles were implanted were stained with blue and red color, indicat-

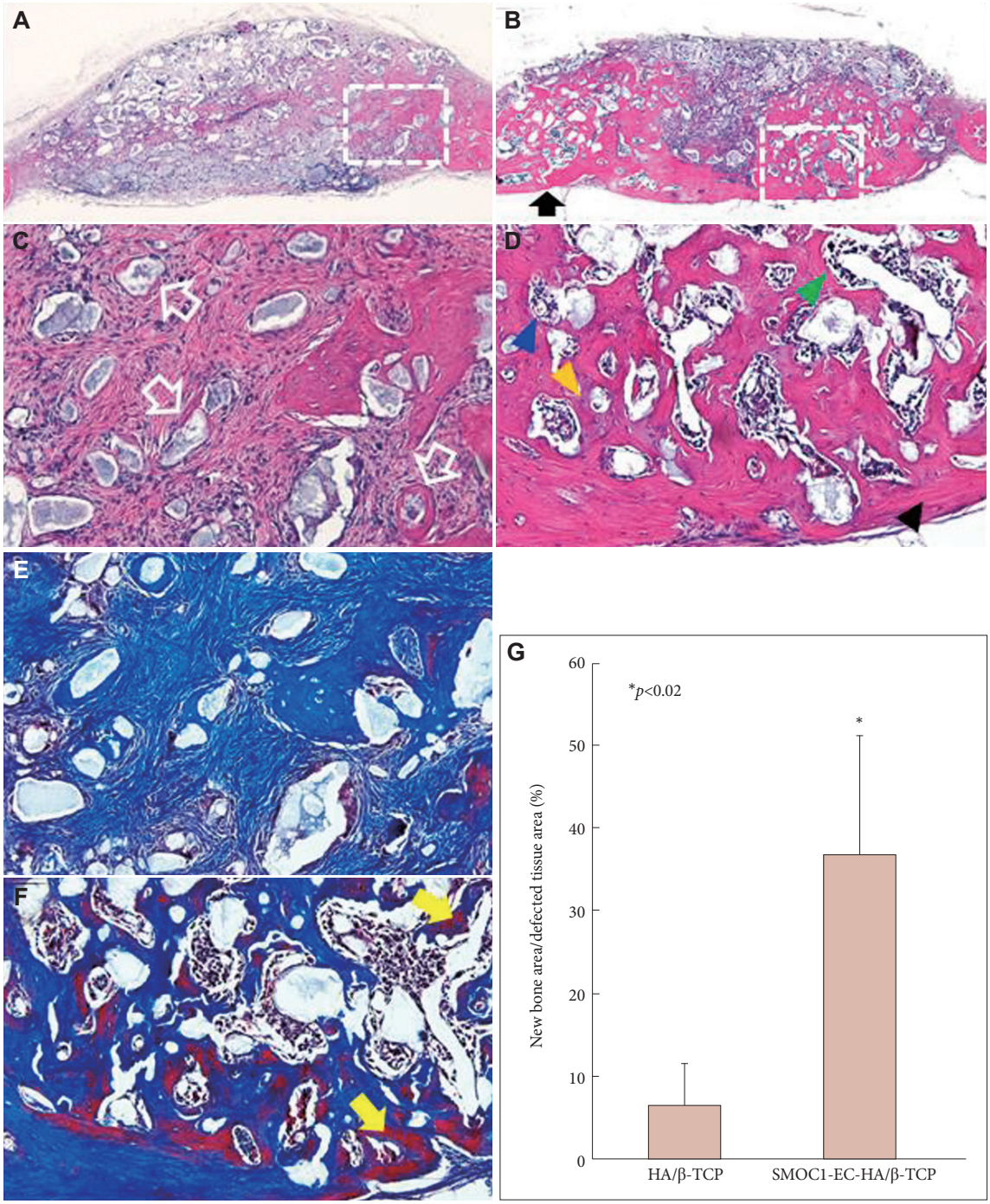


Figure 5. Histology of calvarial defects implanted with SMOC1-EC peptide-immobilized HA/β-TCP. (A-D) H&E staining of implanted calvariae with (A and C) unmodified and (B and D) SMOC1-EC peptide-immobilized HA/β-TCP. (E-G) Masson's trichrome staining of calvariae implanted with (E) unmodified and (F) SMOC1-EC peptide-immobilized HA/β-TCP. The ratio of the area of newly formed bone to the total defected area was measured (n=4, G). SMOC1-EC: SPARC-related modular calcium binding 1-extracellular calcium, HA/β-TCP: hydroxyapatite/β-tricalcium phosphate.

ing that newly formed bone was undergoing maturation (Fig. 5F, yellow arrow). Collectively, these results demonstrate that HA/β-TCP particles coated with immobilized SMOC1-EC peptides efficiently induced new bone formation and maturation in the calvarial defect model.

This is the first report showing that the immobilization of peptides derived from the EC domain of SMOC1-EC onto HA/β-TCP enhances bone regeneration in calvarial defects *in vivo*. Therefore, the surface immobilization of SMOC1-EC peptides may lead to improvements of bone regeneration in clinical applications.

DISCUSSION

Synthetic bioceramics have been widely used as bone substitutes in the field of orthopedics and dentistry. However, unmodified bioceramics contain no bioactive constituents and thus, a great effort has been made to incorporate bioactive substance into ceramics for functional improvement. An array of papers has shown that surface modification of bioceramics with bioactive peptides significantly improves bone regeneration [26]. We have also previously shown that HA/β-TCP modified with MEPE peptides results in superior bone regeneration compared with that of unmodified HA/β-TCP [14]. In addition, immobilized epidermal growth factor (EGF) increases the spreading and survival of MSCs more strongly than soluble EGF does through sustained activation of extracellular signal-regulated kinase signaling [27]. This sustained activation might have been due to the inhibition of internalization and/or the degradation of EGF receptors [28,29]. Arginine-glycin-aspartic acid motif-containing peptide-immobilized ceramics were also reported to stimulate bone regeneration through activation of integrins [15]. In this study, we found that the immobilization of peptides derived from the SMOC1-EC domain onto HA/β-TCP significantly enhanced bone regeneration in calvarial defects. Previously, we reported that SMOC1 is an ECM protein that plays a critical role in the osteogenic differentiation of human BMSCs [30]. We were interested in the EC domain of SMOC1 because calcium is important for biological processes such as mineralization, and the domain is conserved among the SPARC family members. The EC domain of SMOC1 has 34% sequence homology with that of SPARC/osteonectin and mediates the adhesion of epithelial cells to SMOC1 [31]. Moreover, the EC domain of SPARC has been shown to interact with a glycoprotein, myocilin [32]. Recently Kwon et al. [33] reported that myocilin is expressed in MSCs and stimulates their differentiation into osteoblasts by activating MAP kinase signaling pathways. Although sequence homology between the EC domains of SMOC1 and SPARC is not very high, it is still possible that myocilin can interact with the SMOC1-EC peptide and thus activate a cascade of signaling pathways including MAP kinases, resulting in the osteogenic differentiation of human BMSCs. Further study may be necessary to address this issue.

As mentioned earlier, HA/β-TCP lacks organic constituents and is biodegradable and osteoconductive *in vivo*. To improve the functionality of HA/β-TCP ceramics, SMOC1-EC peptides were immobilized to the surface of HA/β-TCP particles through an established protocol using APTES and PEG-$(SS)_2$ [14,34-36]. Although we could not quantitatively analyze the immobilization efficiency, XPS analysis showed that the SMOC1-EC peptides were successfully bound on the surface of the HA/β-TCP particles (Fig. 3).

The implantation of SMOC1-EC peptide-immobilized HA/β-TCP into the calvarial defect region induced substantial bone regeneration compared with that induced by implantation of unmodified HA/β-TCP (Figs. 4 and 5). In the bone tissue of the SMOC1-EC peptide-immobilized HA/β-TCP, blood vessels and lamellar bone were clearly observed (Fig. 5D), indicating that the bone regeneration process was successfully underway. Currently, the mechanism through which immobilized SMOC1-EC peptides stimulate bone regeneration is unclear, but the SMOC1-EC peptides may activate certain cell surface receptors. Previously, it was shown that the EC domain of SMOC1 contains an EF hand calcium-binding motif that was reported to interact with integrins αvβ1 and αvβ6 [37]. Integrins are important for cell communication with components in their microenvironment, such as ECM molecules, and are ubiquitously expressed in most cells, including MSCs [38]. Therefore, SMOC1-EC peptides immobilized onto HA/β-TCP may stimulate integrin signaling, thus inducing the osteogenic differentiation of MSCs and resulting in bone regeneration.

In addition, covalently immobilized molecules may have a longer sustention time after implantation compared with absorbed molecules, and they remain longer on the surface, increasing the cellular response [36]. The immobilized SMOC1-EC peptides may be slowly released from the implanted area along with the biodegradation of HA/β-TCP. Therefore, sustained release of the SMOC1-EC peptide and continuous stimulation of integrins by the peptide may also in part contribute to enhanced bone regeneration. Further study may be necessary to determine the molecular mechanisms underlying enhanced bone regeneration by SMOC1-EC peptide and SMOC1-EC peptide-immobilized HA/β-TCP.

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Conflicts of Interest

The authors have no financial conflicts of interest.

Ethical Statement

This study was approved by the Institutional Ethics Committee of Kyungpook National University.

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