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BBP-Functionalized Biomimetic Nanofibrous Scaffold Can Capture BMP2 and Promote Osteogenic Differentiation

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Abstract

Bone morphogenetic proteins (BMPs, *e.g.*, BMP2 and 7) are potent mediators for bone repair, however, their clinical use has been limited by their safety and cost-effectiveness. Therefore, innovative strategies that can improve the efficacy of BMPs, and thereby, use a lower dose of exogenous BMPs are highly desired. Inspired by the natural interaction between extracellular matrix (ECM) and growth factors, we hypothesize that bone matrix-mimicking nanofibrous scaffold functionalized with BMP binding moieties can selectively capture and stabilize BMPs, and thereby, promote BMP-induced osteogenic differentiation. To test our hypothesis, a gelatin nanofibrous scaffold was fabricated using thermally induced phase separation together with a porogen leaching technique (TIPS&P) and functionalized by a BMP-binding peptide (BBP) through cross-linking. Our data indicated that BBP decoration largely improved the BMP2 binding and retention capacity of the nanofibrous scaffolds without compromising their macro/microstructure and mechanical properties. Importantly, the BBP-functionalized gelatin scaffolds were able to significantly promote BMP2-induced osteogenic differentiation. Moreover, BBP alone was able to significantly stimulate endogenous BMP2 expression and improve osteogenic differentiation. Compared to other affinity-based drug delivery strategies, *e.g.*, heparin and antibody-mediated growth factor delivering techniques, we expect BBP-functionalized scaffolds will be a safer, more feasible and selective strategy for endogenous BMP stimulating and binding. Therefore, our data suggests a promising application of using the BBP-decorated gelatin nanofibrous scaffold to stimulate/capture BMPs and promote endogenous bone formation *in situ* in contrast to relying on the administration of high doses of exogenous BMPs and transplantation of cells.

Graphical abstract

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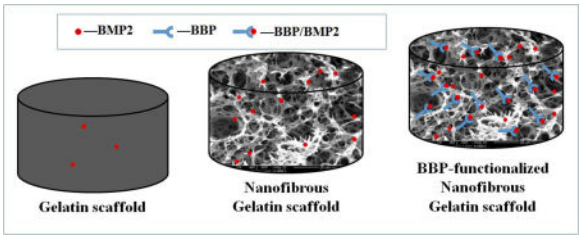

Bone matrix-mimicking nanofibrous gelatin scaffold functionalized with BMP binding moieties can selectively capture and stabilize BMP2, and thereby, promote BMP2-induced osteogenic differentiation.

Keywords

Nanofibrous scaffold; Bone morphogenetic protein; Bone morphogenetic protein–binding peptide; Endogenous bone regeneration; Osteogenic differentiation

1. Introduction

Annually, more than five hundred thousand patients receive bone defect repairs in the United States, with a cost greater than $2.5 billion. This figure is expected to double by 2020 due to several factors, including the needs of the baby-boomer population and increased life expectancy [1, 2]. Repair of large bone defects caused by tumor resection, trauma, infection, and congenital malformation remains a significant unmet clinical challenge. Tissue engineering is a promising alternative to the gold standard, autologous bone graft, which is limited by morbidity at the donor site and difficulties in preparing anatomically-shaped grafts from harvested bone [3–7]. Biomaterials-mediated exogenous stem/progenitor cells transplantation (*e.g.*, bone marrow mesenchymal stem cells, BMSCs) or growth factor/hormone delivery (*e.g.*, bone morphogenetic protein, BMPs) are two widely studied tissue engineering approaches. These approaches, however, have encountered crucial barriers in therapeutic translation. For instance, the Food and Drug Administration (FDA)-approved osteogenic growth factors BMP2 and 7 are carried by absorbable collagen sponges (ACS) demonstrate their validity for bone repairs through recruiting/inducing endogenous stem/progenitor cells for osteogenic differentiation [8–10]. The success of BMP-based therapy, however, has been significantly impeded by several factors. They include super-physiologic dose requirements, high costs, a short half-life, and other serious (and undesirable) side effects, including the potential for ectopic bone formation, inflammation, and increased cancer risk [11–15], which further complicate treatment outcomes.

To address these challenges, several strategies have been proposed and investigated with limited success to date. For example, polymeric nano/microparticles have been developed to incorporate BMPs, and thereby prevent them from degradation and achieve sustained/controlled release by tailoring the polymeric carrier degradation rate [16, 17]. However, technical challenges with these techniques (*e.g.*, high burst release rate, low incorporation efficiency, inappropriate release kinetics, harmful degradation products, protein denaturation during the complex and harsh incorporation process, *etc.*) need to be addressed to improve

their applications [11, 18–20]. With an increased understanding of the role of extracellular matrix (ECM) during tissue repair, researchers are recognizing that the fibrous networks are not only providing mechanical support and adhesion sites to cells, but also acting as a reservoir for many growth factors which control a variety of cellular processes [21]. Particularly, heparin, as a naturally sulfated biopolymer with a high negative charge in ECM, can bind and stabilize positively charged heparin-binding proteins, including many growth factors, *e.g.*, BMPs [22]. Therefore, a lot of effort has been invested in developing heparin-modified materials for protein delivery. These bio-inspired, affinity-based systems have emerged as attractive strategies for delivery of growth factors by mitigating some challenges of the aforementioned controlled release systems [23]. However, the major concerns of using heparin are the potentially severe side-effects, including hemorrhagic complications, such as thrombocytopenia due to its anticoagulant function *in vivo*. Moreover, the difficulty in the modification of heparin-based biomaterials, susceptibility to desulfation, and the batch-to-batch variability make it more undesirable for translational applications [24, 25].

Therefore, innovative strategies to improve the efficacy of BMPs for bone repair are highly desired. Recently, a 19-amino acid cyclic peptide (BBP) derived from bone matrix, has been found to avidly bind to BMP2; thereby, strongly enhancing BMP-induced bone formation [26–32]. Although the mechanism remains elusive, the unique capacity of selectively binding to BMP prevents it from degradation and partially explains the intriguing function of BBP [26–32]. Notably, a series of emerging findings indicates that endogenous BMPs (mainly BMP2), generated at the injury site are enough to heal critical-sized bone defect by transplanting an anti-BMP2-antibody immobilized ACS [33–35]. Although the antibody-based strategy is expensive and difficult for therapeutic translation, these findings underscore the feasibility of promoting critical bone healing by coordinating endogenous BMPs. Compared to BMP antibody, BBP is a small, synthetic peptide that is more stable and relatively inexpensive. Furthermore, the selective affinity for BMPs is also a desirable feature of BBP, which heparin does not display. Therefore, BBP is superior in translational applications compared to other current affinity-based methods. However, there is an outstanding knowledge gap that needs to be addressed before BBP can be further applied for bone regeneration, for example, 1) what is the cellular function of BBP, and 2) if it is feasible to immobilize BBP onto solid-state scaffolds for local BMP2 capturing and functionalization. Therefore, we strive to answer these critical questions in our current studies.

Gelatin, the partially denatured derivative of collagen, is chemically similar to collagen, which is the most abundant protein in the bone organic matrix (>90%). Combining the thermally induced phase separation (TIPS) method with porogen leaching technique (TIPS&P), gelatin nanofibrous (GF) scaffolds can be fabricated and have proven to facilitate osteogenic differentiation and bone formation. Their success can be attributed to their biomimicking physical structure in addition to their similar chemical composition to the native bone matrix [36–38]. It is noted that additional osteogenic signals (*e.g.*, BMPs/osteoprogenitors), are still required, and must be supplemented in scaffolds for successful osteogenic differentiation and bone regeneration [36–38]. Thus, a bone matrix-mimicking scaffold functionalized with BMP binding moieties that can improve BMPs-induced osteogenesis is highly desired. The high surface area of the nanofibrous structure and

abundant functional groups (*e.g.*, –NH2 and –COOH) of GF scaffold makes it favorable for substrate-based drug delivery and further modification (*e.g.*, cross-linking with peptides).

Therefore, our central hypothesis is that biomimetic GF scaffolds can promote BMP2-induced osteogenesis through functionalization with BBP to selectively capture and provide a sustained release of BMP2 for osteogenic induction.

2. Materials and methods

2.1. Preparation of Gelatin Nanofibrous (GF) Scaffolds

GF scaffolds were fabricated by using TIPS&P technique as previously described [36–38]. Briefly, gelatin type B was dissolved in 50% of ethanol/water solvent at 40 °C to make a 5 wt% homogeneous gelatin solution. 2 mL gelatin solution was added into silica vial and then transferred into −80 °C freezer for phase separation overnight. After that, the samples were immersed into ethanol at −20 °C for 24 h and the solvent was changed three times. Then the samples were transferred into 1, 4-dioxane solution for another 24 h with agitation; the solvent was changed three times. At last, the samples were removed from 1, 4-dioxane and frozen at −20 °C overnight and lyophilized for 48 h.

2.2. Chemical Crosslinking of GF with BMP2 Binding Peptide (BBP)

BBP (>98% in acetate) (CRSTVRMSAEQVQNVWVRC-EEE) [27, 31] was commercially synthesized by Biomatik. A carboxyl-rich peptide domain (EEE) was added to the original peptide as a spacer to couple with gelatin. BBP (2 mg) was first dissolved in 50 μl Dimethylformamide (DMF) and then transferred to 1 mL MES buffer ((2-[morpholino]ethanesulfonic acid), pH 5.3), after that 9 mL acetone was added into MES buffer to prepare MES/acetone mixture. GF scaffolds were added to BBP MES/acetone solution and crosslinked with 1-Ethyl-3-(3-(dimethylamino)propyl) carbodiimide HCl / N-hydroxysuccinimide (EDC/NHS) at 4 °C for 24 h. The scaffolds were freeze-dried after a thorough wash. To visualize the distribution of BBP in GF scaffolds, an FITC-labeled scrambled peptide (FITC-QEQLERALNSS, Biomatik) was used as a model peptide and cross-linked onto GF scaffolds.

2.3. Mechanical Test

Mechanical properties of GF and GF-BBP scaffolds were measured with the MTS insight single column 100 N electromechanical testing system purchased from MTS Systems Co. (Eden Prairie, MN) at a crosshead speed of 1 mm/min. The samples were cut into cylindrical shapes with the diameter of 5 mm and the height of 2 mm. The modulus value was determined from the linear region of the acquired stress-strain curve.

2.4. Effect of BBP on BMP2 Retention *In Vitro* and *In Vitro* BMP2 Release

The effect of BBP on retention of BMP2 was studied and analyzed through BMP2 ELISA Development kit (Peprotech, USA). Briefly, 125 ng recombinant human BMP2 (rhBMP2, Peprotech, USA) was coated onto a prepared 2D GF film, 3D GF, and 3D GF-BBP scaffolds and incubated for 1 h at room temperature, the samples were then washed with PBS three times, 10 min each time to remove free rhBMP2. After that rhBMP2 loaded samples were

lysed with RIPA buffer on ice for 10 min and the lysate was collected for ELISA analysis. The amount of rhBMP2 retained on the specimens was determined with the ELISA kit and measured at 405 nm by a microplate reader (Infinite M200, Tecan)

After the GF scaffolds, GF-BBP scaffolds and GF films coated with 125 ng rhBMP2, the samples were washed with PBS three times, 10 min each time to remove free rhBMP2. After that rhBMP2 loaded samples were soaked in 1 mL PBS at 37 °C. The supernatant was collected and replaced with fresh PBS at 18 h, 40 h, 90 h, 114 h, 140 h and 168 h. The amount of rhBMP2 was determined with the rhBMP2 ELISA kit according to the manufacture's instruction and measured at 405 nm by the microplate reader.

2.5. Cell Viability

The multipotent C2C12 cell was a generous gift from Dr. Yifan Li at the University of South Dakota. The cell viability of C2C12 cells (5×10^4 cells per scaffold) on GF, GF-BBP scaffolds was studied on day 1 and 4 by using MTS assay (Promega, USA). GF scaffolds without BBP were selected as control and the cell viability was expressed as 100%.

2.6. ALP Activity

GF and GF-BBP scaffolds were incubated with 200 ng of rhBMP2 for 1 h at room temperature and washed with PBS for three times, 10 min each time. 1×10^5 C2C12 cells were seeded in GF and GF-BBP scaffolds. The cells were cultured for 7 days in DMEM containing 5% FBS with 300 ng/mL of rhBMP2 [39]. For ALP staining, the samples were treated according to the manufacture's instruction (Sigma-Aldrich, Saint Louis, MO). For Quantitative ALP, the samples were lysed with lysis buffer provide by the SensoLyte® pNPP Alkaline Phosphatase Assay Kit (AnaSpec, San Jose, CA) and centrifuged for 10 min at 2500 g at 4 °C. The supernatant was reacted with *p*-nitrophenyl phosphate (*p*NPP), incubated for 30 min at 37 °C and the absorbance was measured at 405 nm. The ALP activity was normalized against total protein content.

2.7. Real-time PCR Analysis

1×10^5 C2C12 cells were seeded into rhBMP2 treated GF and GF-BBP scaffolds. After 7 days of incubation, total RNA of cells was isolated using the GeneJET™ RNA Purification Kit (Thermo Scientific™, Waltham, MA). An equivalent amount of RNA was processed to generate cDNA using the High Capacity cDNA Reverse Transcript kit (Applied Biosystems, Forster City, CA). Real-time PCR was analyzed with the Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA). TaqMan® Gene Expression Assays of GAPDH (Mm99999915), BSP (Mm00436767), OCN (Mm03413826), and BMP2 (Mm01340178) were purchased from Applied Biosystems (Forster City, CA).

In order to study if BBP alone can stimulate osteogenic differentiation, C2C12 were treated with a different dose of BBP with or without rhBMP2 (100 ng/mL) for 7 days. Then the ALP activity and osteogenic gene expression in C2C12 cells were measured as described above.

2.8. Statistical Analysis and Image Editing

To determine the statistical significance of observed differences between the study groups, a two-tailed homoscedastic *t-test* was applied. A value of $p < 0.05$ was considered to be statistically significant while $0.05 < p < 0.10$ was considered to represent a nonsignificant, but clear trend in cell response. Values are reported as the mean ± standard deviation (SD).

3. Results

3.1. Morphology of BBPs-decorated Scaffolds

The 3D GF-BBP scaffolds showed interconnected macroporous structure together with nanofibrous strut morphology (Figure 1 (A, B)), which were similar to the GF scaffolds reported previously [36–38]. To evaluate the cross-linking efficiency and distribution of peptide in GF scaffolds, confocal images of both GF and GF-FITC-Peptide scaffolds were studied after extensive washing. Under the same confocal settings, the neat GF could not emit any autofluorescence (Figure 1C) while the FITC-labeled peptide emitted strong fluorescence (Figure 1D). All of these results demonstrated that the BBP was able to successfully conjugate onto the GF scaffolds through the chemical cross-linking method. Moreover, the GF scaffolds could retain the macro and microstructure after the peptide decoration.

3.2. Mechanical Property of BBPs-decorated Scaffolds

Compression tests were carried out on both cross-linked GF and GF-BBP scaffolds (Figure 2). The general shapes of stress-strain curves for both types of scaffolds were similar and no considerable compression variation was observed. These results indicated that the conjugation of BBP with the scaffold has no significant effect on the mechanical properties of GF scaffold.

3.3. BMP2-retention and Release on BBPs-decorated Scaffolds

In order to study the BMP2 binding ability of BBP-immobilized scaffolds, the retained BMP2 amount on the scaffold was measured by ELISA assay. As the data indicates (Figure 3), a significantly higher amount of rhBMP2 was retained in GF-BBP scaffolds (~20.1 ng per scaffold) after extensive washing when compared to GF scaffolds (~14.2 ng per scaffold) ($p<0.05$) and solid Gel films (~7.9 ng per scaffold) ($p<0.001$), respectively. Interestingly, GF scaffolds exhibited largely higher rhBMP2 retention than solid Gel films ($p<0.01$), which may be due to the high surface area of gelatin nanofibers. In addition to binding significantly higher amounts of rhBMP2, BBP-GF scaffold also exhibited a sustained release of rhBMP2 to the supernatant and maintained a higher concentration of rhBMP2 at each time point, compared to other groups (Figure 3B). During 7 days of release, the total amounts of rhBMP2 released from GF-BBP scaffolds, GF scaffolds, and Gel films were approximately 22.3 ng, 14.9 ng, and 11.2 ng, respectively. The amounts of rhBMP2 remained in GF-BBP, GF scaffold, and Gel film were around 5.0 ng, 3.3 ng, and 1.8 ng respectively. Therefore, the large surface area of the nanofibrous structure together with the high affinity between BBP and rhBMP2 contributed to the improved capacity of GF-BBP scaffolds for BMP2 binding and protection from degradation.

3.4. Cell Viability on GF and GF-BBP Scaffolds

The cell viabilities of C2C12 cells on GF and GF-BBP scaffolds were quantitatively measured by MTS assay after culturing for 1 and 4 days. As shown in Figure 4, GF-BBP scaffolds exhibited slightly higher cell viabilities at both day 1 and 4 ($P < 0.05$). This result demonstrated that the presence of BBP was able to mildly affect C2C12 cell growth on the scaffolds.

3.5. C2C12 Osteogenic Differentiation on GF-BBP Scaffolds

ALP staining results showed that more ALP positive (purple) cells were observed on the GF-BBP scaffolds (Figure 5B) compared to that of GF scaffolds after 7 days of culturing in growth medium (Figure 5A). Consistent with the staining data, quantitative measurements indicated cells cultured on GF-BBP scaffolds expressed significantly higher ALP activity compared to the ones on GF scaffolds (Figure 5C).

In addition to ALP activity, Real time PCR results (Figure 6) also showed that cells cultured on GF-BBP scaffolds expressed significantly higher levels of osteogenic genes, including BSP and OCN compared to the cells cultured on GF scaffolds. Therefore, these data indicated BBP-functionalized GF scaffolds (GF-BBP) were able to significantly improve exogenous rhBMP2 induced osteogenic differentiation.

3.6. Effects of BBP on BMP2-induced Osteogenic Differentiation

The osteogenic property of BBP was further studied in rhBMP2 untreated and treated C2C12 cells. The ALP activity exhibited a dose-dependent manner with the increase of BBP concentration (Figure 7). Similarly, rhBMP2 treated groups also exhibited elevated ALP expression with the increase of BBP concentration. Although the fold change in BBP-only treated groups was not as high as the response of rhBMP2 BBP-containing groups, this data still suggests that BBP alone can promote the osteogenic differentiation of C2C12 cells.

In addition to ALP activities, gene expression on BBP and rhBMP2-BBP groups was investigated (Figure 8). BBP concentration of 5 μg/mL and rhBMP2 concentration of 100 ng/mL were chosen. Consistent with ALP activity, the quantitative gene expression results also indicated that BBP and BMP-BBP could promote osteogenic gene expression, including BMP2 and OCN. It is interesting to note that the BBP-only group exhibited a higher BMP2 expression compared to BMP-BBP group. These data indicated that BBP may maintain the stability of exogenous BMP2 and promote the expression of endogenous BMP2.

4. Discussion

BMPs (*e.g.*, 2 and 7) are probably the most potent inducers of osteogenic differentiation and act as the key regulators during embryogenesis, bone formation, and repair [40–42]. It has been suggested that the clinical efficacy of recombinant BMPs depends highly on the carrier system/materials. The main role of a delivery system for BMPs is to ensure an effective delivery and retention of adequate protein concentrations to the desired site, providing an initial support for cell recruitment, growth, and differentiation to bone tissue [43, 44].

Although a variety of materials have been tested for BMPs delivery, collagen sponges are still the most widely used delivery materials in clinical applications [45]. However, the clinical efficacy of BMPs is still low, and even though the retention of transplanted proteins *in situ* has been improved by using collagen sponges, limitations of collagen-based materials still exist [46]. Thus, smart/biomimetic biomaterials/delivery systems are still needed for the increasingly urgent clinical needs of BMPs applications in orthopedic and dental areas [40].

Since the collagen currently used is derived from animal tissues, the animal-derived materials create concerns about the risks of immunogenicity and pathogen transmission [47]. Fortunately, the safety concerns associated with collagen can be largely circumvented by using gelatin instead. Gelatin is a denatured biopolymer derived from collagen and has an almost identical chemical composition as that of collagen [48]. In addition to lower immunogenicity compared to collagen, gelatin also showed higher protein retention and the ability to improve BMPs-induced osteogenic differentiation and bone formation [49]. Moreover, the high tunability and low cost enable gelatin to be a versatile polymer for biomedical applications. It has been recognized that nanofibrous scaffolds with connected macropore structure that mimic the natural ECM are favorable for osteoblastic differentiation and bone formation [50–52]. Although our data confirmed that the GF scaffolds can non-specifically absorb a small amount of rhBMP2, the affinity of BMPs to gelatin scaffolds is still limited due to the absence of the BMP-binding moieties. It was noted that the BBP-functionalized GF scaffolds showed the highest ability for retention and sustained release of rhBMP2 compared to both the gelatin films (without either nanofibrous structure or BBP moieties) and GF scaffolds (without BBP moieties). This suggested both the high surface area of nanofibrous structures (quickly released from non-specific absorption) and the BMP binding moieties (relatively slowly released from specific BMP binding) contributed to the improved features of the functionalized scaffolds. Importantly, BBP-functionalized GF can not only capture more rhBMP2, it can also significantly improve the rhBMP2-induced osteogenic differentiation on the scaffolds compared to the GF scaffolds. Recently, another group developed a self-assembling peptide amphiphile (PA) nanofibrous hydrogel to improve rhBMP2 functions by conjugating with a synthetic BMP-binding peptide [53]. Although the BBP peptide sequence and the scaffold are different from our studies, their data also indicated that BBP-functionalized gel scaffolds can improve rhBMP2 binding affinity and its induced osteogenic differentiation [53], suggesting it is a promising strategy to improve BMP-based therapy by functionalizing the scaffolds with BMP binding moieties. Whereas, our innovative BBP-decorated GF scaffold more closely mimicked the functions of bone matrix with strong binding affinity to rhBMP2 in addition to the similarity in chemical composition and physical structure, compared to the previous report [53].

Our studies not only demonstrate the feasibility of using the BBP to functionalize the solid-state scaffolds instead of the hydrogel, but also reveal some new mechanisms by which BBP promotes rhBMP2-induced osteogenic differentiation. Although previous studies proved that BBP was able to significantly improve both rhBMP2 and rhBMP7 induced-bone formation in different *in vivo* models (possibly because the soluble BBP could strongly bind to BMPs and thereby stabilize them *in vivo*) [26–32], it was not known if BBP could be conjugated to nanofibrous scaffolds while retaining its BMP binding ability. In order to reduce any

potential interference from the gelatin substrate, we added a carboxyl-rich peptide domain (EEE) as a spacer between the original BBP and the main gelatin structure. Our data indicated that the BBP-conjugated gelatin matrix was able to effectively capture and release rhBMP2, suggesting the BBP remained functional after cross-linking to the scaffolds. In contrast, we did not find the improved BMP2 binding affinity when we examined another BBP using the same conjugation strategy, which was reported to be effective in the PA gel system [53]. Furthermore, other than the BMP binding affinity, the mechanism by which BBP improves BMP-induced bone formation is still elusive. Our cellular experiments indicated, for the first time, that BBP can not only promote exogenous rhBMP2-induced osteogenic differentiation, (possibly through the known binding mechanism) but also can significantly stimulate the endogenous BMP2 expression and induce osteogenic differentiation in C2C12 cells without exogenous rhBMP2. Although further studies need to be completed to fully understand the intracellular molecular mechanisms, our results revealed a new and exciting contribution of BBP osteogenic differentiation. We chose C2C12 as the cell model to investigate the rhBMP2 activity largely because of its high sensitivity to BMP-induced osteogenic differentiation. Actually, as a multipotent myoblast cell line, C2C12 has been widely used in the studies of osteogenic capacity of BMPs *in vitro* [54–56]. The high responsiveness to BMPs treatment may attribute to the robust expression of BMPR-IA on the surface of C2C12 [57]. Therefore, C2C12 is an ideal cell model for testing BMPs activity *in vitro* although other cell types, *e.g.*, primary cells, may provide some valuable and complementary information.

It is intriguing to develop novel biomaterials with strong intrinsic osteoinductivity, thereby, either reducing the dosage of exogenous BMPs or avoiding their use entirely. Although polymer-based scaffolds (both natural and synthetic polymers) have tremendous advantages in bone tissue engineering applications, poly-hydroxyethyl methacrylate (Poly-HEMA) is the only osteoinductive polymer that has been reported to date [58]. Although the exact mechanism of osteoinductivity of biomaterials is still elusive, it will be of great interest to develop innovative biomimetic polymeric biomaterials by targeting the essential endogenous signals (*e.g.*, BMPs) for bone regeneration. Our most recent studies proved that the hypoxia-mimicking GF scaffold could promote cranial bone regeneration without the addition of exogenous cells or BMPs, potentially through the stimulation of endogenous BMPs and angiogenic signals *in situ* [59]. Our current findings suggest that it is possible to promote endogenous bone formation by the BBP-functionalized biomimetic GF scaffolds through the stimulation, capture and sustained release of endogenous BMPs *in situ*, although a higher amount of BBP may need to be incorporated on the scaffolds to achieve satisfactory bone formation *in vivo*.

5. Conclusions

In this work, GF scaffolds with selective and strong rhBMP2 binding affinity were successfully prepared through chemical cross-linking with a BMP-binding peptide (BBP). The BBP-conjugated GF scaffold showed enhanced retention and prolonged release profile of rhBMP2. Importantly, both BBP and the GF-BBP scaffolds showed enhanced BMP2-induced osteoblast differentiation *in vitro*. Notably, our data also revealed that BBP alone was able to significantly stimulate endogenous BMP2 expression and improve osteogenic

differentiation in a dose-dependent manner, in addition to enhancing the osteoinductive ability of exogenous BMP2. Therefore, our data demonstrates the feasibility of improving the osteogenic capacity of exogenous BMP2 by functionalizing the biomimetic GF scaffold with a potent BBP. Moreover, our studies also suggest a promising strategy of using the BBP-decorated GF scaffold to stimulate/capture endogenous BMPs and promote endogenous bone formation *in situ*, in contrast to relying on transplantation of high doses of exogenous BMPs/cells.

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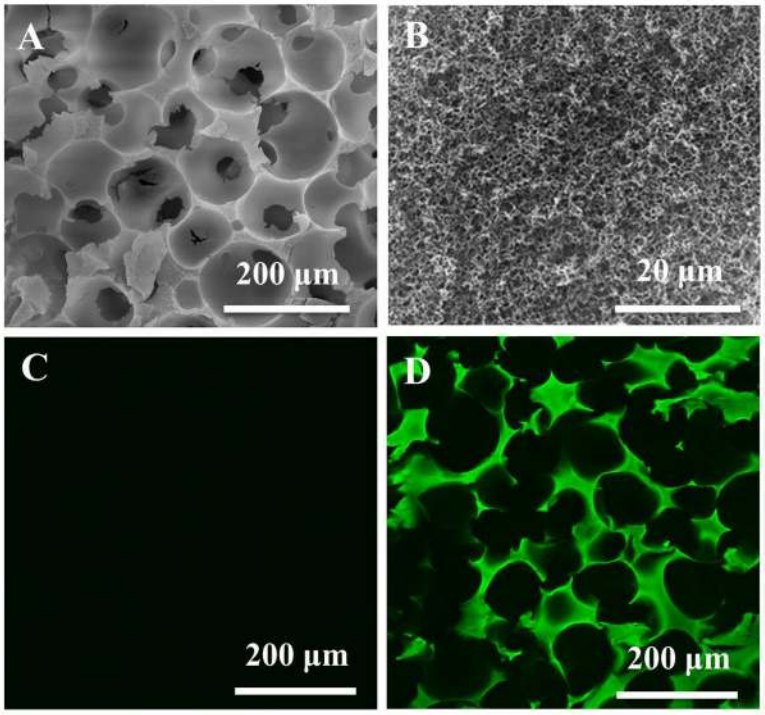

Figure 1.
SEM images of (A, B) 3D GF scaffolds and Confocal images of (C) GF scaffold, (D) GF-FITC-Peptide scaffold.

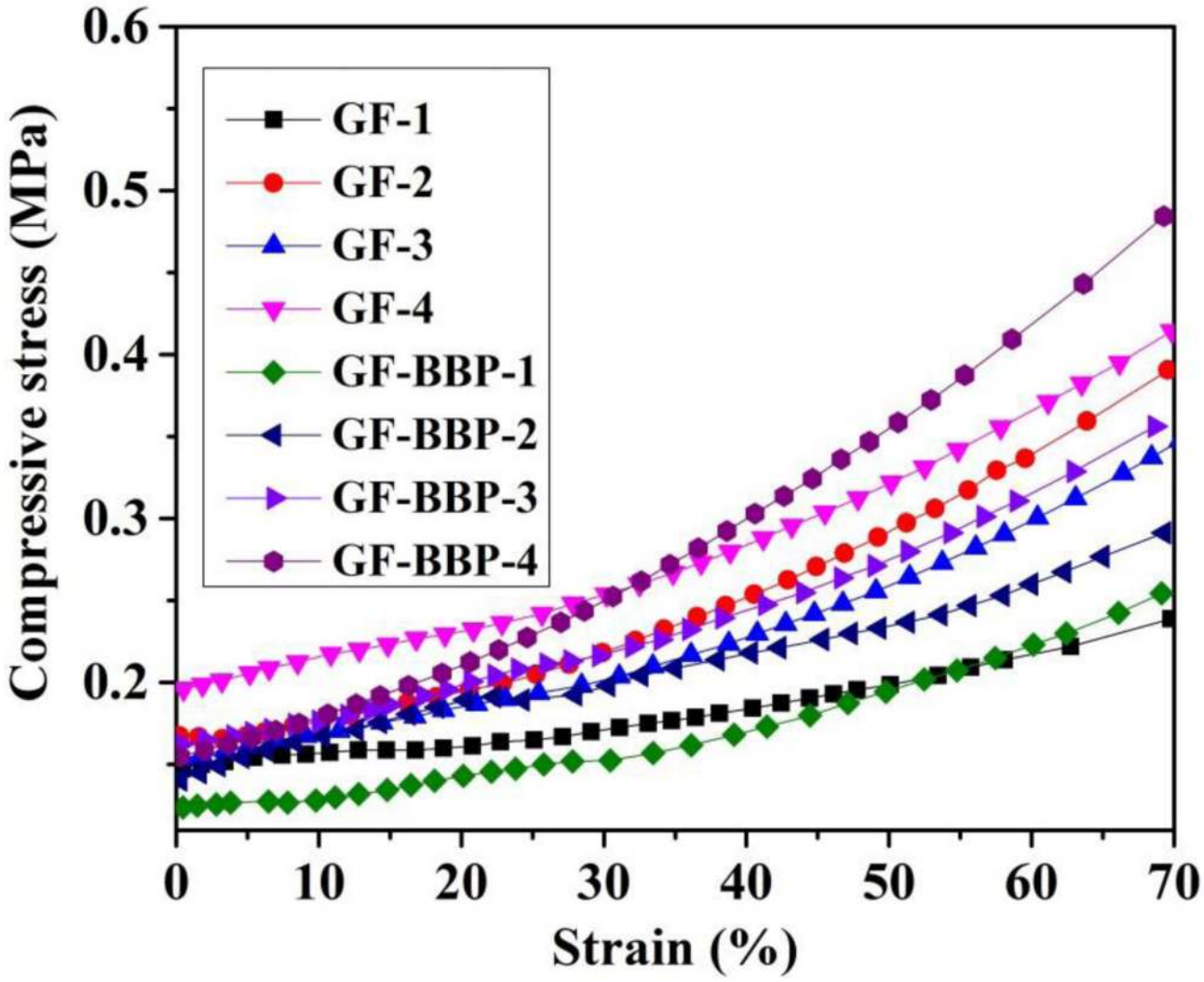

Figure 2.
Representative compressive stress-strain curves acquired from GF and GF-BBP scaffolds (n=4).

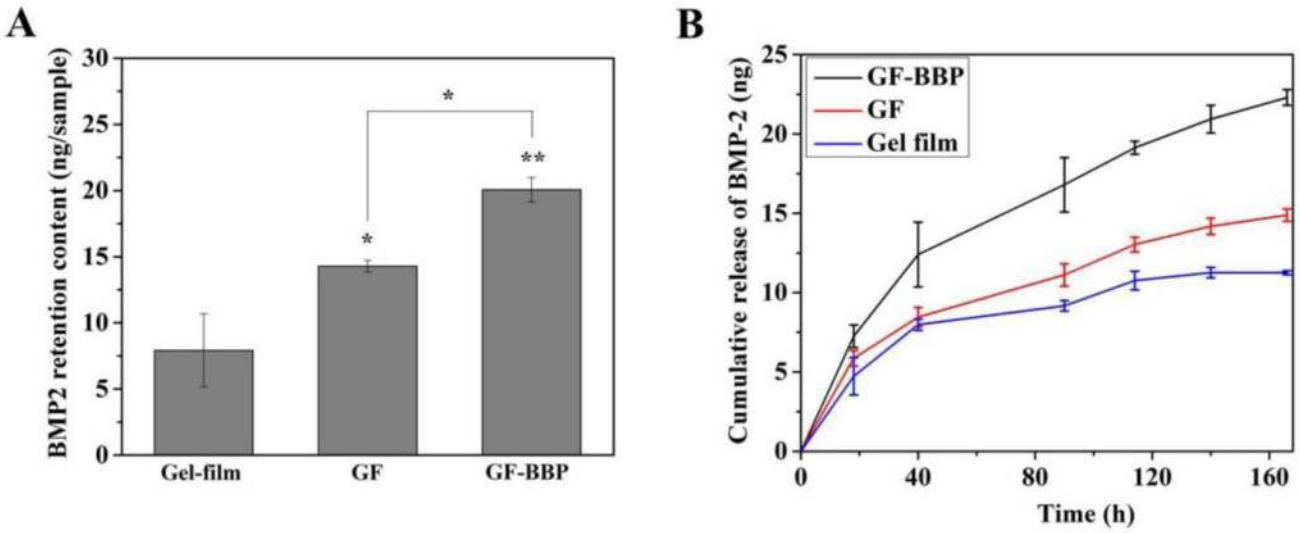

Figure 3.

BMP2-retention and release on BBPs-decorated scaffolds. (A) rhBMP2 retained per scaffold after several extensive washes, (B) *In vitro rh*BMP2 release behavior of samples in PBS at 37 °C for a period time (n=3). Data are expressed as mean ± SD (*$p < 0.05$, **$p < 0.01$).

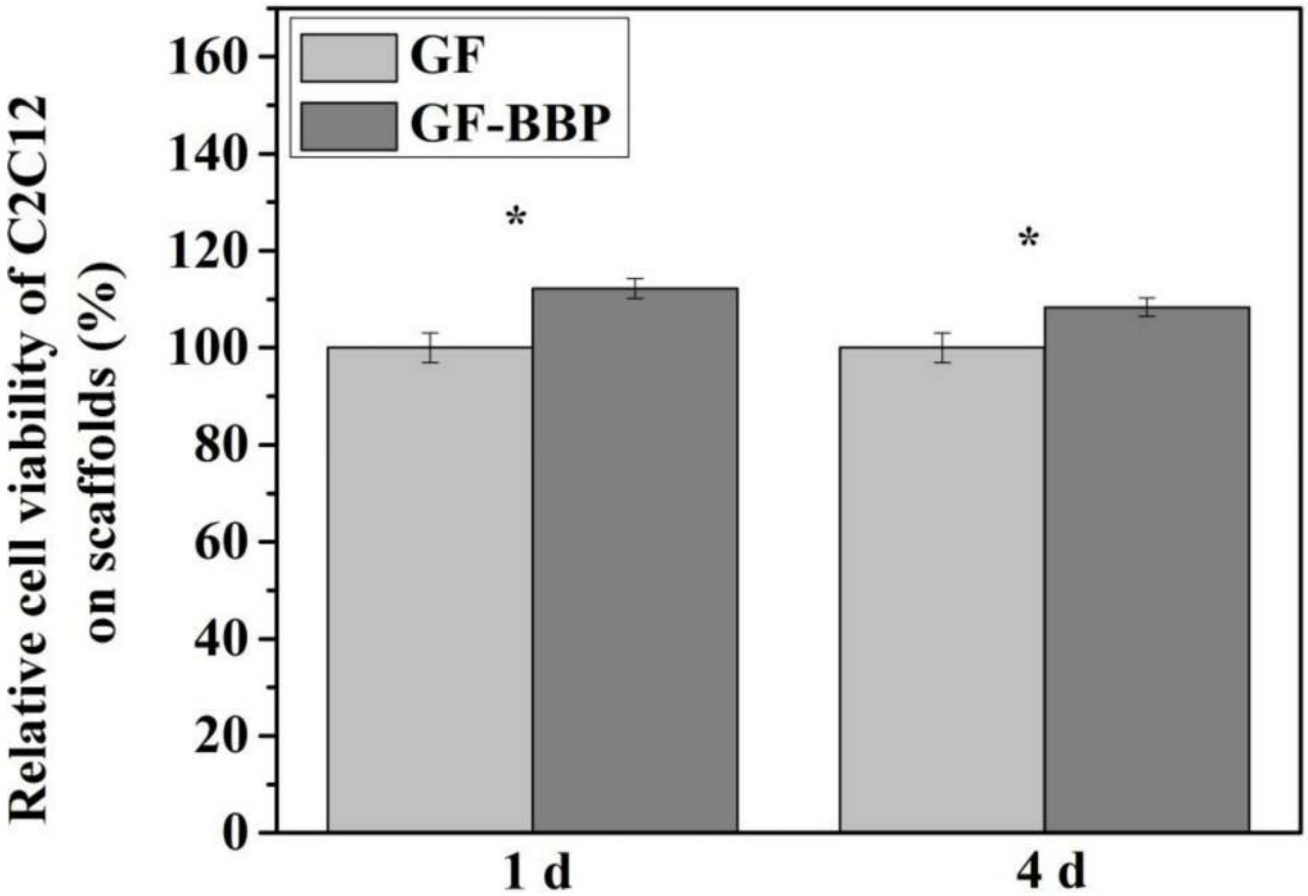

Figure 4.
Cell viability of C2C12 cultured on GF and GF-BBP scaffolds (n=3). Data are expressed as mean ± SD (*$p < 0.05$).

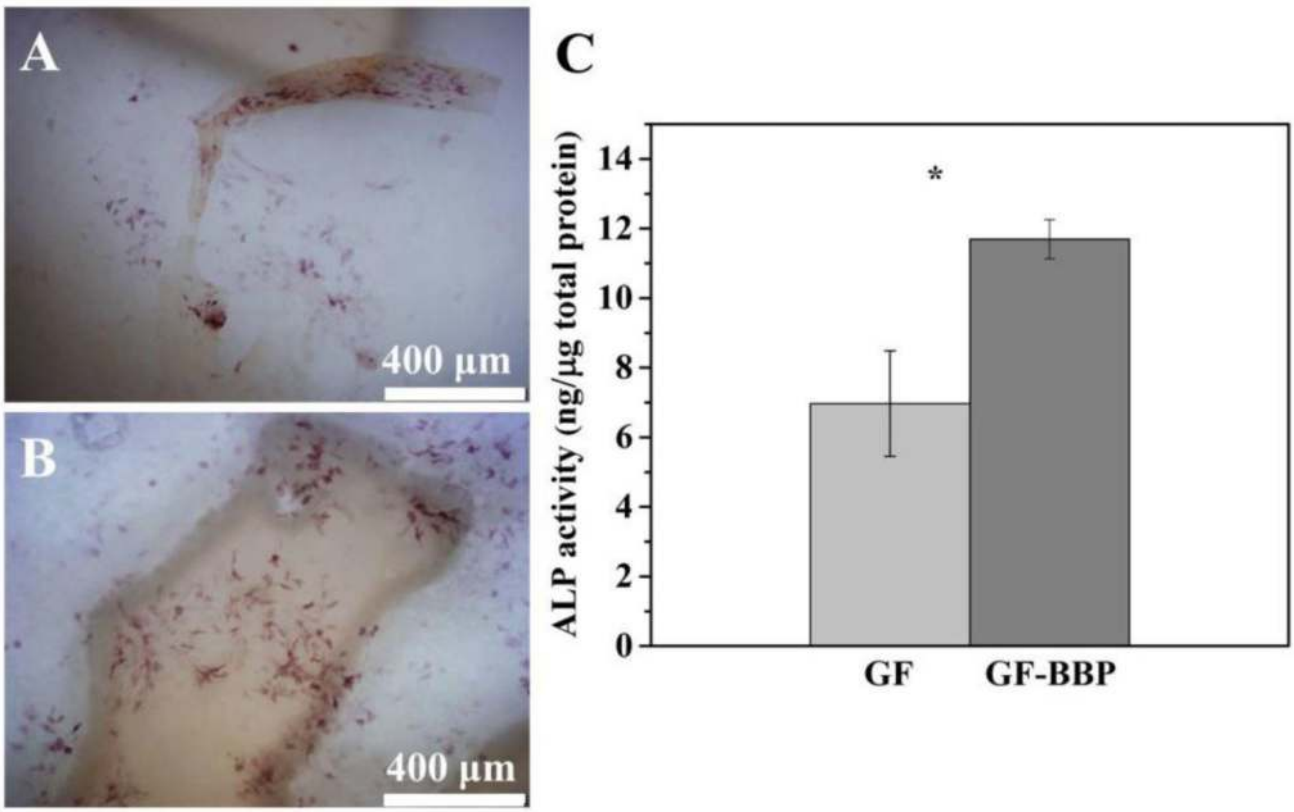

Figure 5.

ALP activity of C2C12 cells on GF (A) and (B) GF-BBP scaffolds. (A) and (B) are ALP staining results while (C) is the quantitative measurement. Data are expressed as mean ± SD (*$p < 0.05$).

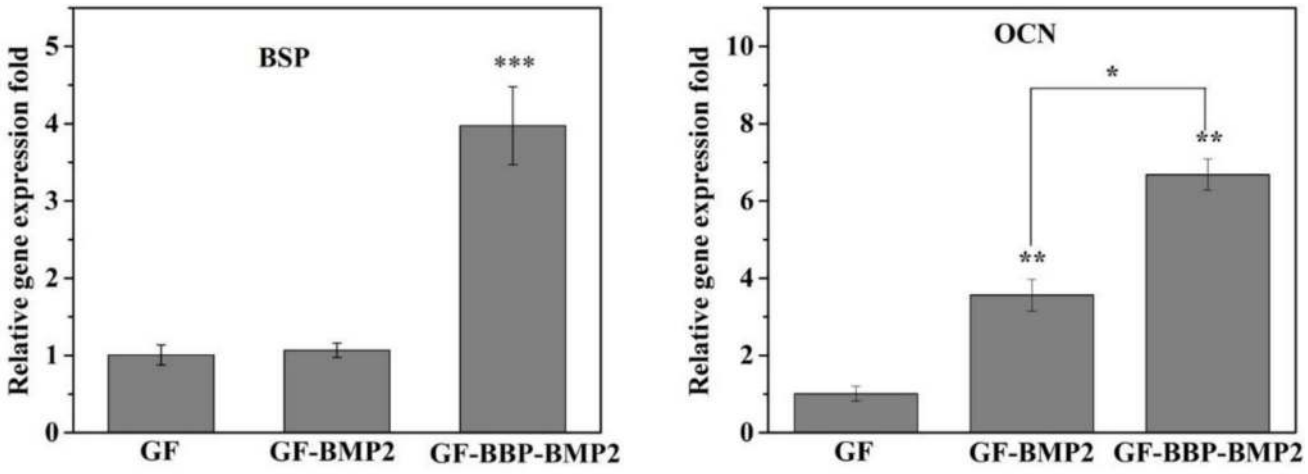

Figure 6.

Relative gene expression of C2C12 cells cultured on GF, GF-BMP2 and GF-BBP-BMP2 scaffolds. Data are expressed as mean ± SD (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

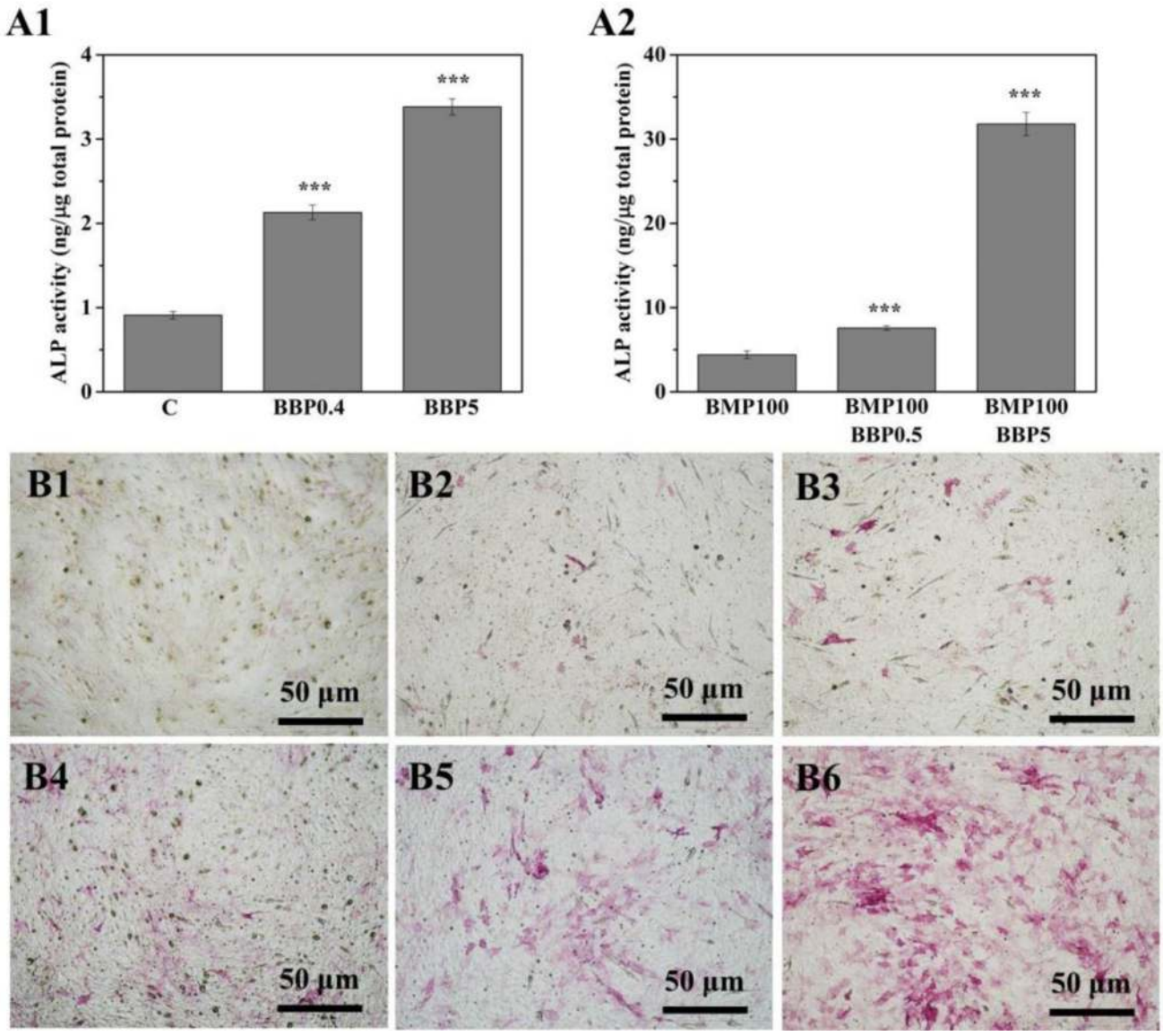

Figure 7.

ALP activities of C2C12 cells after treatment with different concentrations of BBP alone (BBP, A1), or combined with 100 ng/mL of exogenous rhBMP2 (BBP-BMP, A2); ALP staining of control (B1), BBP0.4(B2), BBP5(B3), BMP100(B4), BBP0.5-BMP100 (B5) and BBP5-BMP100 (B6) (Scar bar=50 μm). Data are expressed as mean ± SD (***$p < 0.001$).

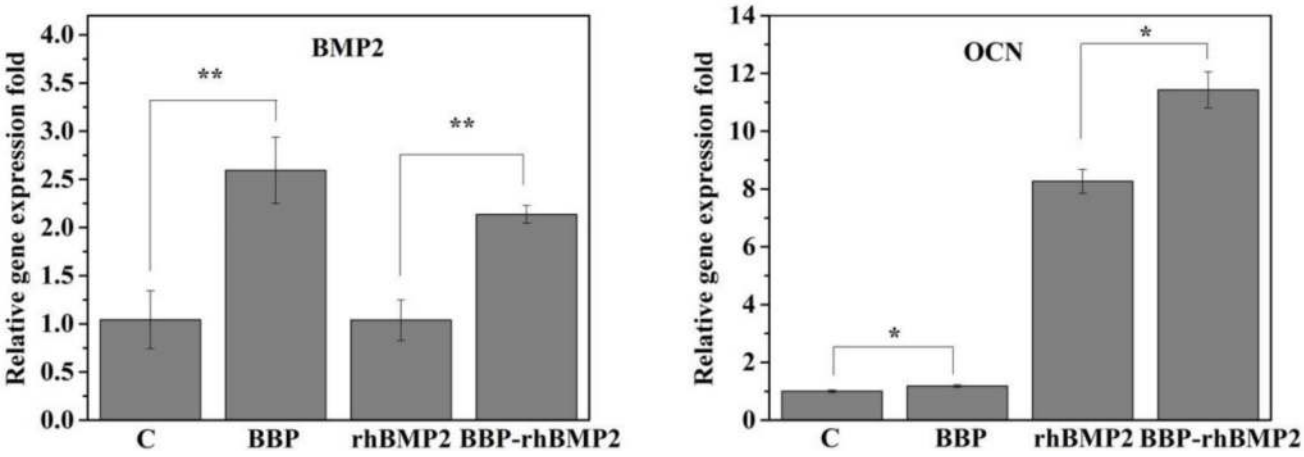

Figure 8.

Relative osteogenic gene expression in C2C12 cells was studied at day 7 after treated with BBP alone (BBP), or combined with 100 ng/mL of exogenous rhBMP2 (BBP-rhBMP2). Data are expressed as mean ± SD (*$p < 0.05$, **$p < 0.01$).