Osteogenic/Angiogenic Dual Growth Factor Delivery Microcapsules for Regeneration of Vascularized Bone Tissue

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Growth factors (GFs) are major biochemical cues for tissue regeneration. Herein, a novel dual GF delivery system is designed composed of poly(lactic-co-glycolic acid) (PLGA) nanoparticles (NPs) and alginate microcapsules (MCs) via an electrodropping method. While bone morphogenetic protein (BMP)-2 is encapsulated in the PLGA NPs, vascular endothelial growth factor (VEGF) is included in the alginate MCs, where BMP-2-loaded PLGA NPs are entrapped together in the fabrication process. The initial loading efficiencies of BMP-2 and VEGF are 78% ± 3.6% and 43% ± 1.7%, respectively. When our dual GF-loaded MCs are assessed for in vitro osteogenesis of umbilical cord blood-derived mesenchymal stem cells (UCB-MSCs) on 2D and 3D environment, MCs contribute to much better UCB-MSCs osteogenesis as confirmed by von Kossa staining, immunofluorescence (osteocalcin, collagen 1), calcium content measurement, and osteogenic markers expression. In addition, when dual GF-encapsulated MCs are combined with collagen and then applied to 8 mm diameter rat calvarial defect model, the positive effects on vascularized bone regeneration are much more pronounced; micro computed tomography (CT) and histology analyses exhibit 82.3% bone healing coupled with 12.6% vessel occupied area. Put together, current study indicates a synergistic effect of BMP-2/VEGF and highlights the great potential of dual GF delivery modality (PLGA NPs-in-MC) for regeneration of vascularized bone.

1. Introduction

Vascularized bone tissue regeneration for the treatment of various bone defects still remains extremely challenging.[1] Therapies using genes,[2] stem cells,[3] and growth factors (GFs)[1,4] are major strategies employed by researchers to induce vascularization during new bone formation and to overcome some drawbacks associated with conventional treatment modalities, such as the use of autografts, allografts, and xenografts. Since a report of dual GF-mediated angiogenesis by Richardson et al.,[5] several groups have examined the effect of dual GF delivery on the regeneration of complex tissues such as angiogenesis,[6] chondrogenesis,[7] myogenesis,[8] osteogenesis,[9–13] and neurogenesis.[4] In particular, studies regarding dual GF-mediated osteogenesis have yet to provide any conclusive findings; while several works reported on an augmented level of osteogenesis via dual GFs delivery,[12–15] others reported on an insignificant level of osteogenesis.[16–18] For instance, Kanczler et al. demonstrated the interplay of osteogenic (bone morphogenetic protein-2; BMP-2) and angiogenic GFs (vascular endothelial GF; VEGF) brings augmented bone formation than that achieved by BMP-2 alone.[13] Similarly, Patel et al. found enhanced bone bridging and union but no significant difference on bone formation by BMP-2 and VEGF than by BMP-2 alone in rat cranial defect.[17] In addition, Kempen et al. reported combination of BMP-2 and VEGF produces synergistic effect on ectopic bone formation but not on orthotopic bone formation.[18] Hence it is widely recognized that selection of appropriate GFs, doses, and delivery system is a very important strategy in achieving...
successful regeneration of vascularized bone tissue. However, the rationale behind such combination of specific GFs and carrier system is still unclear and poorly defined in many instances, due mainly to the complexity of biological functions of GFs in vivo and difficulty in controlling GFs release rate.

Since many investigations have alleged some controversial effects of single or dual GFs delivery on osteogenesis and/or bone healing, this study intends to systemically investigate the benefits of sustained in situ delivery of dual GFs for osteogenesis of umbilical cord blood-derived mesenchymal stem cells (UCB-MSCs) in a 2D and 3D culture environment and more importantly for a vascularized new bone formation via a rat calvarial bone defect model. To do this, we design and prepare a dual GF carrier system for sustained delivery of BMP-2 and VEGF. There are many candidates in fabricating drug delivery systems, including synthetic polymers, biopolymers, and inorganic compounds. Among them, we choose alginate and poly(lactic-co-glycolic acid) (PLGA) for their proven efficacy and versatile advantages.\(^6,20–22\) Alginate is very useful and easy to handle for encapsulation of bioactive molecules, cells, or micro/nanoparticles. PLGA is a biodegradable polymer and very flexible in fabricating micro/nanoparticles. Our dual GF delivery platform is composed of alginate microcapsules (MCs), which include PLGA nanoparticles (NPs); BMP-2 is encapsulated in the PLGA NPs and VEGF is entrapped into the alginate matrix. Our electrodropping system has been very useful in producing uniform sized MCs. The set up of the electrodropping apparatus is simple that allows continuous and automated production of MCs. In addition, this technique offers high production yield, increased encapsulation efficiency, and minimal aggregation of MCs.\(^{21–25}\) Among the previous works, there are few equivalents to our platform in which a dual GF(VEGF/BMP-2) delivery system was fabricated via electrodropping method. The combined use of dual GF delivery system and UCB-MSCs for vascularized bone regeneration is also notable in this work, due to the clinical applications of UCB-MSCs in stem cell therapy.

We hypothesize that in situ delivery of dual GFs should have an added or synergistic effect on the osteogenic differentiation of UCB-MSCs in vitro and more importantly on vascularized new bone formation when delivered in vivo. In general, VEGF and BMP-2 are considered to be a common and potent combination for vascularized bone tissue regeneration. Previous report manifests VEGF secretion happened at the earlier healing stage of bone fracture, whereas BMP-2 secretion during entire fracture healing process.\(^{26}\) To this end, it would be very interesting to see the results that compared to single GF (BMP-2)-loaded MCs, dual GF-loaded MCs could significantly enhance calvarial bone regeneration at the interfacial area between host and transplant as well as in the bulk defect region, along with a notable volume of vascularization. This study clearly indicated a synergistic impact of two different GFs on the regeneration of vascularized bone tissue and demonstrated the role of carrier system that enables sustained delivery of dual GFs from MCs at the target site.

2. Results and Discussion

2.1. Preparation and Characterization of MCs

A GF delivery system of core–shell MCs was proposed in the previous study.\(^6\) However, fabrication of homogeneous core–shell MCs using electrodropping method depends on various parameters including physicochemical properties of polymers, coaxial needle set up, electrostatic repulsion, and flow rate.\(^{20}\) Herein, a new MC design is attempted via PLGA NPs and alginate for dual GF delivery. BMP-2-loaded PLGA NPs are produced (Figure 1A) by employing an ultrasonication-assisted double emulsion evaporation method.\(^{27}\) The size and surface charge of the PLGA NPs were found to be 138 ± 53 nm and −18 ± 0.9 mV, respectively. Scanning electron microscope (SEM) and atomic force microscope (AFM) images of PLGA NPs showed a homogeneous

Figure 1. Dual GF-loaded microcapsules (MCs) fabrication and characterization. A) BMP-2-loaded PLGA NPs exhibit a fairly homogenous distribution of spherical particles, as assessed via B) SEM and C) AFM. D) BMP-2-loaded PLGA NPs and VEGF are encapsulated into alginate to generate dual GF-loaded MCs for the sequential release of GFs (arrows). E) The dual GF-loaded MCs are examined via gross appearance and F) optical microscopy, which indicate the presence of PLGA NPs (black spots). G) Fluorescence of rhodamine red-stained PLGA NPs in the MC and H) SEM image of MC. I) The initial loading efficiency of BMP-2 and VEGF, respectively and J) the release profile of two different growth factors out of the dual GF-loaded MCs as observed for 28 d in vitro.
distribution of spherical particles (Figure 1B,C). Their surface roughness was 29 ± 3.5 nm. The synthesized PLGA NPs were then encapsulated into alginate MCs to carry out sustained delivery of dual GFs. Alginate was chosen due to its versatile carrier of GF, protein, peptide, and drugs and for tissue engineering applications. It is generally accepted that NPs not significantly hamper the bioactivity of BMP-2. In fact, the resulting dual GF-loaded MCs were investigated for GF release kinetics via a transwell system. The release profiles demonstrate the independent GFs release kinetics. The release efficiency of BMP-2 after fabrication of MCs was examined using enzyme-linked immunosorbent assay (ELISA). Beginning with 500 ng of each GF, the initial loading efficiency of BMP-2 in PLGA NPs and VEGF in alginate was 78% ± 3.6% (392 ± 18 ng) and 43% ± 1.7% (216.5 ± 8.8 ng), respectively (Figure 1I).

In addition, the resulting dual GF-loaded MCs were investigated for GF release kinetics via a transwell system. The release profiles demonstrate the independent GFs release kinetics. The release efficiency of BMP-2 and VEGF from the MCs in a given time period (28 d) was found to be 69% ± 5% (270 ± 20 ng) and 59% ± 5% (128 ± 11 ng), respectively (Figure 1I). Early burst release of ~23% BMP-2 (88 ng) and ~23% of VEGF (37 ng) was observed during first 3 d, followed by ~34% of BMP-2 (134 ng) and 25% of VEGF (25 ng) release between day 4 to 14. Overall, the MCs continued the release of both GFs for up to 28 d and exhibited a different release kinetics, in which BMP-2 was released at a higher concentration than VEGF. It should be addressed at this point that both loading efficiency and release profile are indirect evidences regarding BMP-2 bioactivity when delivered from PLGA NPs. Direct evidences can be obtained from in vitro and in vivo experiments. The following results suggest that current fabrication process of MCs is acceptable, not significantly hamper the bioactivity of BMP-2. In fact, polymer NPs and MCs have been widely utilized as a delivery carrier of GF, protein, peptide, and drugs and for tissue engineering applications. It is generally accepted that NPs and MCs not only increase the stability of loaded GFs but prolong the half-life of GFs. In addition, they can be integrated with a transplantable 3D scaffold for targeted tissue regeneration.

2.2. Induction of 2D Osteogenesis

The effect of single or dual GF release from the MCs on the induction of 2D osteogenic differentiation of UCB-MSCs was evaluated using four different groups (Figure 2A). The level of osteogenesis was examined after 14 and 28 d of osteogenic induction in vitro, respectively. All four groups showed positive black stains of mineralized area as assessed via von Kossa staining at 28 d (Figure 2B); quantification of the area positively stained was 66.5% ± 6% (G1), 80% ± 6% (G2), 71.6% ± 7% (G3), and 81% ± 7% (G4) (Figure 2G). Immunofluorescence staining (IFS) results (Figure 2D,E) of osteogenic protein markers, osteocalcin (OCN), and collagen type 1 (Col 1) yield similar results as that of von Kossa staining. Figure 2F shows a merged image of 4′,6-diamidino-2-phenylindole (DAPI; Figure 2C), and IFS of OCN and Col 1. Quantitative analysis of OCN (Figure 2H) and Col 1 (Figure 2I) fluorescence indicates the following order of osteogenic efficiency: G4 ≥ G2 > G3 > G1. In particular, positive staining of OCN was significantly higher among the cells treated with dual GF-loaded MCs (G4) than those incubated with a single GF (G3) (Figure 2H). Additionally, when each group was assessed for osteogenic gene expression, bone sialoprotein (BSP), Col 1, OCN, and osteopontin (OPN) were significantly overexpressed in groups of G2, G3, and G4 compared to G1 (Figure 2J). While not much difference in expression level was observed among G2, G3, and G4, the one of BSP was higher in G4 than in G3. Also, the average expression levels of OPN, and Col 1, while not significant, were greater in G4 than that observed in the other groups. Each group was also assessed for osteogenesis via calcium content measurement (Figure 2K). As expected, the normalized calcium content for the groups supplied with GFs (G2, G3, and G4) was larger than that of G1. In addition when gene expression levels and calcium content were evaluated after 14 d of culture, the outcomes were similar with those observed at 28 d (Supporting Information, Figure S1). Overall, the results of 2D osteogenesis prove that our MCs sustainably release bioactive dual GFs for at least 28 d to promote osteogenesis of UCB-MSCs on 2D culture. It should be noted that the cumulative BMP-2 release from current MCs platform is approximately 270 ng BMP-2, a little over half of that supplied exogenously via medium for G2 (500 ng). The present results coincide with the report of Zhang et al., where they demonstrated the synergistic osteogenic response of adipose-derived stem cells (ADSC) via dose-dependent VEGF and BMP-6 combined delivery than via VEGF or BMP-6 delivery alone. They claimed the synergistic effect was due to a cross talk between VEGF and BMP signaling pathway for the enhancement of osteogenesis.

2.3. Induction of 3D Osteogenesis

It is well known that 2D cell culture does not take into account the complexity of biological, physical, and mechanical properties of natural cellular microenvironment. Hence, we have fabricated a 3D collagen scaffold disc (approximately 8 mm Ø and 2 mm thickness) encapsulating both UCB-MSCs and MCs (Figure 3A). Unlike 2D cell culture, collagen scaffold provides cells with an excellent extracellular microenvironment, wherein UCB-MSCs are able to proliferate, interact with the surroundings, and respond to the released dual GFs from MCs, in 3D. White spots inside the collagen scaffold depict the distribution of MCs (Figure 3B), and an interconnected fibrous network of
the collagen is observed via AFM (Figure 3C). This collagen scaffold is supposed to provide 3D microenvironment for UCB-MSCs and to retain bioactivity of GFs within the scaffold. A cross-sectional SEM image clearly delineates MCs (depicted as stars) embedded in the collagen matrix (Figure 3D). Proper mixing at 4 °C was essential during scaffold fabrication to ensure the distribution of cells and MCs; PKH67 staining confirmed the presence of UCB-MSCs in the collagen scaffold (Figure 3E) and the broad distribution throughout the scaffold at the center (Figure 3F) and in the periphery (Figure 3G). The black cavities correspond to the MCs and the green spots correspond to PKH67-stained cells. As illustrated in Figure 4A the level of osteogenesis of UCB-MSCs in collagen scaffold was analyzed after 28 d of in vitro culture. Low and high magnification (Figure 4B) images of von Kossa stains confirmed the presence of mineralized area for all groups. Quantification of the stained areas was measured to be 6.5% ± 0.6% (G1), 13.7% ± 0.8% (G2), 14.7% ± 0.9% (G3), and 17% ± 0.9% (G4) (Figure 4G). IFS results (Figure 4D,E) of osteogenic protein markers OCN and Col 1 yield a similar trend as that of von Kossa staining. Figure 4F shows a merged image of DAPI, and IFS of OCN and Col 1. Quantitatively compared, the order of osteogenic efficiency: G4 > G3 > G2 > G1 (Figure 4H,I). Additionally, when osteogenic gene expression was assessed by real-time quantitative polymerase chain reaction (qPCR; Figure 4J), all markers were significantly upregulated in G4 than in other groups. However, less osteogenic activity was observed from G2 compared to that of G3 or G4, due mainly to certain role of VEGF on osteogenesis[31] and poor diffusion of BMP-2 into the interior of the scaffold when provided exogenously.[32] While VEGF is a potent angiogenic inducer, it is reported to stimulate osteoblast differentiation and believed to support a
Figure 3. Characterization of collagen scaffolds containing UCB-MSCs and dual GF-loaded MCs. A) UCB-MSCs and dual GF-loaded MCs are prepared as a homogenous mixture and encapsulated in collagen matrix. B) Gross appearance of the resulting collagen scaffolds and C) AFM, in which the topography image shows a fibrous collagen structure. D) The cross-section of the collagen scaffolds is examined via SEM, and exhibits the presence of MCs (stars) within collagen matrix. E) PKH67-stained UCB-MSCs (green) are well distributed within the collagen scaffold as confirmed by fluorescence microscopy. In particular, the black cavities correspond to the MCs while the green spots correspond to PKH67-stained cells. F) Inner and G) peripheral region of collagen scaffold.

synergistic effect of osteogenesis along with BMP-2. Each group was also assessed for calcium content (Figure 4K). The normalized calcium content was much higher in groups using MCs (G3 and G4) and the difference was statistically significant. Meanwhile, the results of gene expression and calcium content analyzed at 14 d were also similar to those at 28 d (Supporting Information, Figure S2). Weinand et al. reported that controlled delivery of BMP-2 via gelatin hydrogel microspheres within 3D scaffold promoted augmented osteogenesis of stem cells derived from human adipose (hASCs) and human bone marrow (hBMSCs). The main interest was to compare the osteogenic potentials between hASCs and hBMSCs in vitro; the role of microspheres and its characterization were not properly addressed. Gabbay et al. exhibited a significant enhancement of osteogenic differentiation of ADSCs while cultured in 3D scaffold than that cultured in 2D monolayer. Taken together, similarly to 2D osteogenesis, it is notable that better osteogenic responses observed in G4 than in G3, most likely due to the positive effect of dual GFs within the 3D scaffold.

2.4. Vascularized Bone Regeneration

After demonstration of dual GF effect on osteogenesis in vitro, we considered a critical size (8 mm) rat calvarial defect model to demonstrate the capacity of our dual GF-loaded MC system for the regeneration of vascularized bone tissue. Studies were carried out for 28 and 56 d, and the results were assessed via micro CT, IFS, SEM, and histology. Temperature-sensitive rat tail collagen was served as a scaffold with a diameter of 8 mm and a thickness of 1.5 mm. A composite disc of collagen and dual GF-loaded MCs was successfully prepared in an implantable form (Figure 5A). The experimental groups were set as G1 (defect only), G2 (BMP-2 loaded into collagen), G3 (BMP-2 delivered from single GF-loaded MCs), and G4 (BMP-2/VEGF delivered from dual GF-loaded MCs) (Figure 5B). Images of the calvarial defect site as assessed via X-ray and micro CT indicate a significant difference of bone regeneration at 28 d (Supporting Information, Figure S3) and at 56 d postoperation (Figure 5C,D). In particular, qualitative bone formation in G4 was significantly better than that observed in G1, G2, and G3. G1 group remains mostly empty supporting that the 8 mm sized defect is critical and self-healing is impossible. Quantification of the data was determined as bone volume (mm³) and percentage of bone regeneration measured within a given region of interest (Figure 5E,F). A higher level of bone regeneration was observed when BMP-2 was delivered from MCs (G3) rather than from the collagen (G2), suggesting that BMP-2 released from MCs was bioactive and more effective in bone healing. More interestingly it was clear that G4 showed the highest levels of bone volume and regeneration, followed by G3, G2, and G1, in decreasing order (Figure 5E,F). This result strongly hints a synergistic effect of dual GFs that VEGF may play a certain role in promoting a bone healing process. In addition, bone regeneration by dual GF in MCs-loaded scaffold was also evaluated via von Kossa staining (Figure 6A); the most mineralized deposit was detected in G4, followed by G3, G2, and G1 as quantitatively determined (Figure 6D). The protein levels of osteogenesis were also examined via IFS for OCN and Col 1 for all groups, quantification of the fluorescence signals demonstrates the following osteogenic trend: G4 > G3 > G2 > G1 (Figure 6E,F). Specifically, G4 yields three and six fold higher levels of OCN and Col 1 expression, respectively, than those of G1. G4 also displays much higher expression of OCN than G2 and G3. Taken together, these findings imply that the mode of GF delivery is crucial and impact of dual GFs is very positive on current calvarial bone healing. Given that the dual GF-loaded MCs (G4) promoted the highest level of bone regeneration, the role of VEGF on vascularized bone regeneration is a particular interest in this study because active vascular network is a prerequisite for successful functional bone regeneration. In fact, VEGF is a well-known angiogenic factor and plays an important role in angiogenesis within bony bridging to achieve vascularized bone regeneration. VEGF not only actively participates in the development of the vascular system but also plays a role in promoting angiogenesis in bone. Therefore, primary interest is posed on the level of vascularization of our transplants in the calvarial defect models. The thin-sectioned samples were stained with H&E for histological analysis (Figure 7A–C). The H&E stains of sagittal section of entire calvarial bone appear in Figure 7A. High magnification images taken at the interface region (marked in blue arrows) between newly regenerated bone and host bone tissue are also obtained (Figure 7B). A significant difference at the interfacial bone healing was obvious; a nearly full integration between host tissue and transplants was notable only with G4 as assessed by tissue thickness and interconnectivity. Upon the inclusion of dual GF-loaded MCs, bone regeneration was significantly better than that of single GF-loaded MCs.
examination of cell penetration into the transplants, it seems that cells migrated and mostly localized in the upper side of the transplant, forming a rich vasculature in the bony bridges (Figure 7C).

Interestingly this phenomenon was not as pronounced in G1, G2, and G3. Dual GFs may consequently stimulate cell migration (chemotaxis) toward the transplants from the surrounding tissues and cell populations are much dense in G4. It is unclear at this time about which GF is more responsible for cell migration. Previous studies have suggested a dose-dependent chemotactic effect of VEGF on primary human osteoblasts, human mesenchymal progenitor cells, and osteoclasts.[31] Quantification of vessel density and vessel occupied area indicates that G4 has approximately three- to four-fold higher levels than those of G3, G2, and G1 (Figure 7D,E). In addition, when mature blood vessel markers von Willebrand factor (vWF) and α-smooth muscle actin (α-SMA) was examined via IFS (Figure 8A), the highest level of vWF and α-SMA expression was noticed in G4 as quantitatively assessed (Figure 8E,F), followed by G2, G3, and G1, in decreasing order. SEM was also used to further analyze the development of new vessels; Figure 8B exhibits a whole cross-sectional view of each group. Figure 8C,D shows the SEM images of peripheral and inner region of the sections, respectively. The presence of red blood cells (RBCs) (marked as red arrows; blue triangles and lines indicate the upper boundary area of each sample) was confirmed in all groups. In particular,
new blood vessels, found in the peripheral regions, were the most rich in G4 (Figure 8 C). Furthermore, the amount of blood vessels observed around the MC was highest in G4, possibly due to the VEGF released from the MCs. Overall, on average 82.3% of bone healing (Figure 5 F) and 12.6% of vessel occupied area (Figure 7 E) was accomplished via the use of dual GF-loaded MCs (G4). The present calvarial data strongly demonstrate the positive effect of dual GFs on vascularized bone healing. We speculate that VEGF and BMP-2 may work in tandem to initiate and develop a vascularized new bone tissue. It should be however noted that this study is insuffi cient in delivering a mechanistic insight of dual GF delivery system on vascularized bone regeneration. It is thus highly recommended that further study need to focus more on mechanistic point of view.

3. Conclusions
This study is supposed to address the effect of our unique dual GF delivery system on osteogenesis of UCB-MSCs in vitro and vascularized bone regeneration in vivo. Alginate MCs containing VEGF and BMP-2 loaded PLGA NPs were successfully fabricated in a well-controlled manner. Tested in four different groups, it was interesting that the positive effect of dual GFs delivery on in vitro osteogenesis of UCB-MSCs was more pronounced in 3D environment, rather than in 2D, as confi rmed via von Kossa staining, IFS, osteogenic markers expression, and calcium content measurements. In addition, using rat calvarial bone defect model, micro CT images, histology, and IFS revealed a signifi cant improvement of neovascularization as
Figure 7. Histology (H&E staining) of the newly formed bone in a calvarial defect model. Rat calvarium samples are examined via low and higher magnifications of H&E stained images. The whole defect region of the calvarium is shown in A). While the interface region between the host bone and newly regenerated bone is marked in blue arrows B), the newly formed bone in the defect region is shown in C). Black arrows indicate red blood cells detected in a vascular structure. In addition, D) microvessel density and E) vessel occupied area within the defect site are also quantified using the histological images. Results are mean ± SD of triplicate measurements and are representative of three independent experiments. ***p < 0.001 and **p < 0.01 indicate a statistically significant difference, respectively.

Figure 8. Vascularization in the newly formed bone for a calvarial defect model. Rat calvarium samples are examined via A) DAPI staining (blue) for the nucleus, IFS of von Willebrand factor (vWF, green), and α-smooth muscle actin (α-SMA, red). The regenerated defect sites are also analyzed via B) low magnification SEM images and higher ones for the C) peripheral and D) inner defect regions. Red arrows indicate red blood cells within a vascularized region (scale bar: 50 μm). The blue triangles and lines suggest the upper boundary area of each sample. Positively stained areas are quantified for E) vWF and F) α-SMA, respectively. Results are mean ± SD of triplicate measurements and are representative of three independent experiments. ***p < 0.001, **p < 0.01, and *p < 0.05 indicate a statistically significant difference, respectively.
well as bone healing after 56 d postoperation; 82.3% of bone healing and 12.6% of vessel occupied area were noticed when treated only with dual GF-loaded MCs. Taken together, this study demonstrates that our dual GFS (VEGF and BMP-2) platform via PLGA NPs-in-MCs is a very effective delivery modality in promoting a synergistic effect on the regeneration of vascularized bone tissue.

4. Experimental Section

Dual GF-Loaded MC Fabrication: PLGA NPs were loaded with 500 ng of BMP-2 (R&D System; Minneapolis, MN) in 1% bovine serum albumin (BSA) using a modified protocol. Briefly, PLGA NPs were prepared via an ultrasonication-assisted double emulsion evaporation method (Sonim Dismembrator Model 500, Fisher Scientific) using an oil phase of 6% w/v PLGA (75:25, RG 757S, MW: 270 000; Boehringer Ingelheim, Germany) and an aqueous phase of 5% w/v polyvinyl alcohol (PVA; 87.6–89.6% hydrolyzed, MW 13,000–23,000; Aldrich). Simultaneously, 500 ng of VEGF (R&D System; Minneapolis, MN) in 1% BSA was added into a 1:1 (v/v) mixture of aqueous sodium alginate (1% w/v; 300–400 cp; Wako Chemicals, Osaka, Japan) and ethylene glycol (Sigma-Aldrich, St. Louis, MO). The PLGA NPs and alginate solution were then mixed thoroughly prior to MC fabrication. An electrodripping system (NanoNC, e5-robot electrosprining spray system, Seoul, Korea) was used under the following settings to prepare the dual GF-loaded MCs: 28G needle gauge, 120 μM min−1 Row rate, 11 kV applied electric voltage, and 10 cm distance between the nozzle tip and grounded collector (Supporting Information, Figure S4). The electrodripped MCs were immediately solidified in 1% w/v CaCl2 solution, sequentially washed with 1% w/v PKH-67 (Sigma, USA). A disposable three-way stopcock (HS-T-01, Gibco BRL, Grand Island, MD) was used under the following settings to prepare the dual GF-loaded MCs: 28G needle gauge, 120 μM min−1 Row rate, 11 kV applied electric voltage, and 10 cm distance between the nozzle tip and grounded collector (Supporting Information, Figure S4). The electrodripped MCs were immediately solidified in 1% w/v CaCl2 solution, sequentially washed with 1% w/v PKH-67 (Sigma, USA). A disposable three-way stopcock (HS-T-01, Gibco BRL, Grand Island, MD) was used under the following settings to prepare the dual GF-loaded MCs: 28G needle gauge, 120 μM min−1 Row rate, 11 kV applied electric voltage, and 10 cm distance between the nozzle tip and grounded collector (Supporting Information, Figure S4).

Characterization: A PLGA NP emulsion was spin-coated onto a freshly cleaved mica substrate while stored MCs were placed on a glass substrate and dried in vacuum oven at 37 °C. Subsequently, field emission scanning electron microscopy (FE-SEM; Joel 7500F, USA) and bio-AFM (NanoWizard II, JPK Instruments, Berlin, Germany) equipped with a HYDRA2R-50NC AFM probe (AppNano) were utilized to obtain morphological images of the particles. The average size and distribution of the particles were analyzed using a laser diffraction particle sizer (Malvern Mastersizer 2000, Malvern, UK). Rhodamine red-stained PLGA NPs were encapsulated in MCs to evaluate the distribution pattern of PLGA NPs inside alginate MCs. Finally, root mean square roughness (Rq) and stiffness (E, Young’s modulus) of the MCs were analyzed using a BioAFM-based microindentation method, following a previous report. The total amount of BMP-2 and VEGF loaded into the PLGA NPs in alginate MCs, respectively, was measured using an enzyme-linked immunosorbent assay (ELISA; R&D Systems). The CaCl2 solution after MC solidification was collected and tested for determination of the loss of GFS. The initial loading efficiency (%) was calculated, based on the ratio between initial content of GF and lost amount of GF. To assess GF release, once dual GF-loaded MCs were put into 8 μm pore size Falcon cell culture inserts (Becton Dickinson Labware, 35–3182, France), and stored at 4 °C for future use.

Preparation and Characterization of Collagen Scaffold Encapsulated with MCs and UCB-MSCs: Type I collagen, extracted from rat tail following a standard protocol, was prepared in acetic acid, diluted with 1×, 10× Medium-199 (Lonza) and neutralized with 1 M NaOH. GF-loaded MCs were homogeneously mixed with 0.3% (w/v) collagen solution, followed by addition of UCB-MSCs (1 × 104) prestained with cell tracker dye PKH-67 (Sigma, USA). A disposable three-way stopcock (HS-T-01, Hyup sung Medical Co., Ltd, Korea) was employed to thoroughly mix the MCs and UCB-MSCs with collagen. The entire procedure (Supporting Information, Figure S5) was carried out in an ice bath to prevent the gelation of collagen. The mixture was placed in the mold and allowed for gelation under a standard culture condition (37 °C, 5% CO2) for 30 min, subsequently cultured in minimum essential medium alpha (α-MEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S; Gibco BRL, Grand Island, MD). The distribution and viability of the PKH67-stained cells was imaged using fluorescence microscopy (CKX41-F32FL; Olympus, Japan). A collagen scaffold was photographed using a digital camera (TG-2; Olympus, Japan). Finally, SEM (Phenom G2 pro desktop, Eindhoven, Netherlands) images of the scaffold were obtained with fixing with 4% paraformaldehyde, dehydrated through a graded series of ethanol, embedded in paraffin, and sectioned into 5 μm thick slices. They were then sputter-coated with platinum, and photographed via SEM.

Induction of 2D Osteogenesis In Vitro: UCB-MSCs (passage 8–10) were seeded at a density of 2.5 × 104 cells cm−2 and cultured in α-MEM supplemented with 10% FBS and 1% P/S. To investigate the osteogenic potential of MCs in vitro, 50 mg of MCs in 8 μm pore size Falcon inserts were placed on six-well plates containing UCB-MSCs, and cultured in osteogenic medium – α-MEM supplemented with 10% FBS, 1% P/S, 10−7 M ß-glycerophosphate, 0.1 × 10−6 M dexamethasone, and 50 μg mL−1 L-ascorbic-2-phosphate – under standard culture conditions (37 °C and 5% CO2). The experimental groups were set as G1 (without BMP-2), G2 (50 ng BMP-2 added exogenously in every medium exchange that accounts for 500 ng during 28 d), G3 (≈270 ng BMP-2 delivered from single GF-loaded MCs), and C4 (≈270 ng BMP-2=128 ng VEGF delivered from dual GF-loaded MCs). The medium was changed every 2 d and the UCB-MSCs were incubated for either 14 or 28 d. At the end of each time point, the samples were washed with phosphate buffered saline (PBS), fixed in 4% p-formaldehyde, and stored for subsequent osteogenic analysis.

Induction of 3D Osteogenesis In Vitro: A mixture of collagen, UCB-MSCs, and MCs with or without GFS were prepared as previously stated. The same initial loading amount of GFS as used on 2D was also applied. The collagen scaffolds were incubated in osteogenic medium for either 14 or 28 d under standard culture conditions (37 °C and 5% CO2), and the medium was changed every 2 d. At the end of each time point, the scaffolds were retrieved, washed with PBS, fixed in 4% p-formaldehyde, dehydrated through a graded series of ethanol, embedded in paraffin, serially sectioned into 5 μm thick sections, and stored for subsequent osteogenic analysis.

Measurement of Calcium Content and Total Protein Amount: Calcium content and total protein amount are determined after 14 and 28 d to assess the osteogenic potential of MCs. 2D UCB-MSC cultures were treated with 0.5% Triton X-100 solution, scraped, collected, and vortexed for 30 min. Meanwhile 3D collagen scaffolds were added with 0.5% Triton X-100 solution and ultrasonicated using a probe tip sonicator for 10 min for scaffold homogenization. Calcium content and total protein amount were then measured using a Quantichrom Calcium Assay Kit (DICA-500, BioAssay systems, USA) and a bichononic acid (BCA) assay kit (Pierce), respectively, following the manufacturer’s instructions. Samples were measured for their absorbance using a micro plate reader (Multiskan spectrum, Thermo Scientific).

Real-Time Quantitative Polymerase Chain Reaction (qPCR): Osteogenic differentiation of UCB-MSCs was evaluated by examining the expression levels of osteogenic gene markers, alkaline phosphatase (ALP), BSP, Coll 1, OCN, OPN, and runt-related transcription factor 2 (RUNX2). Total RNA was isolated from each sample using TRIZOL (Gibco BRL, NY, USA) reagent, followed by determination of the quantity and quality of each RNA sample via a Nanodrop ND-1000 spectrometer (Thermo Scientific, Wilmington, DE). Subsequently, cDNA templates were synthesized using a Maxime RT PreMix kit (Intron, Seoul, Korea) according to the manufacturer’s instructions. Upon completion, 1 μL of cDNA from each sample was mixed with Real MOD Green (Intron, Seoul, Korea) and amplified via an ABI 7500 Real-Time PCR system (Applied Biosystems, CA, USA). Gene expression...
levels of each sample were analyzed via 2−ΔΔCT method. The primers used are as follows: ALP: CCACGTCTTCACATTTGGTG (forward), GACCTGGGCTCTTCTCAG (reverse); β-actin: GGCTCTCCAGAACATCATCC (forward), TTTCTAGACGGCAGGTCAGG (reverse); ALP: CCACGTCTTCACATTTGGTG (forward), GACCTGGGCTCTTCTCAG (reverse); GAPDH: TGCACTGGGTCATGTGTTTG (forward), TGGCTGCATTGAAAAGACTG (reverse); RUNX2: AGCAGAGCGACACCCTAGAC (reverse); OPN: TGAAACGAGTCAGCTGGATG (reverse); OCN: GGCAGCGAGGTAGTGAAGAG (forward), GATCTTCTGGGGTGGTCTCA (reverse); Col 1: TGGCCTGTCATCGTGGT (forward), TGTGAAAATTCATGGCTGTGG (reverse); collagen peptides in the form of a gelatin sponge. The samples were stained with Alizarin Red S for calcium detection.

**Histology and SEM:** Samples were subjected to hematoxylin and eosin (H&E) and von Kossa staining following a standard protocol. Calcium deposits appeared as black or brownish-black spots and images were taken using optical microscopy (Carl Zeiss Axios Vert.A1 Microscope, Germany). Sagittal in vivo sections were dehydrated through a graded series of ethanol (50% to 100%), dried completely, sputter-coated with platinum, and imaged via SEM to analyze the formation of bone vessels within the defect region. Multiple regions within the defect sites were analyzed for the presence of RBCs and used to quantify vessel density (Image J). Quantitative analysis was performed using Image J software (NIH). The H&E-stained sections (two slices per defect, three microscopic fields per ROI) were photographed and used to quantify the mean vessel density, and mean vessel occupied area as assessed by the position of RBCs.

**Immunofluorescence Staining of Angiogenic Protein Markers:** Angiogenic markers, α-SMA and vWF were assessed. A one-way analysis of variance (ANOVA) with Tukey’s multiple comparison post hoc test was conducted using a commercial software (GraphPad Prism 5, San Diego, USA). Statistically significant difference was determined as * (p < 0.05), ** (p < 0.01), and *** (p < 0.001), respectively.

**Supporting Information**

Supporting Information is available from the Wiley Online Library or from the author.

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