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Brasenia-inspired hydrogel with sustained and sequential release of BMP and WNT activators for improved bone regeneration

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ABSTRACT

Although bone morphogenetic protein (BMP) and WNT signaling play pivotal roles in bone development, homeostasis, and regeneration, the applications of proteins to stimulate corresponding signaling pathways showed limited outcomes in the repair and regeneration of bone defects that might be attributed to the reciprocal interventions of these pathways. In order to satisfy the combinational and sequential activation of BMP and WNT pathways, inspired by the heterogeneous hydrogel-liked structures of *Brasenia*, heterogeneous alginate/chitosan hydrogels were fabricated and spatially loaded with FK506 and BIO to achieve sustained and sequential release of the activators. Alkaline phosphatase staining, alizarin red staining and qRT-PCR results suggested that FK506 and BIO enhanced osteoblastic differentiation *in vitro* when used separately. Besides, by mixing and matching the activators and the hydrogel layers, a superior releasing mode that a combination of early FK506 release and following BIO release was identified *via* both *in vitro* and *in vivo* explorations for most efficient bone regeneration. These results suggested that drug-loaded heterogeneous hydrogels possess great potentials in treating bone loss defects for future clinical practice.

Bone loss caused by fracture, tumor, osteoporosis, *etc.*, is a common clinical problem, which may seriously impact our life quality. Amongst the various approaches developed to repair bone loss, the gold standard is autogenous bone transplantation. However, there still some problems exist, such as limited bone mass and secondary trauma [1]. In contrast, using tissue engineering strategies to regenerate bone has a capacity to overcome those challenges [2], and therefore, has become one of the hotspots in the past several decades.

Bone morphogenetic protein (BMP) is widely used due to its strong capability in osteogenesis, and is currently the only commercially available therapeutics that serves as an alternative to bone grafts [3–5]. However, it is shown that the clinical BMP application in the treatment of bone loss could lead to undesired outcomes and a failure of the treatment. Besides ectopic bone formation and inflammation, it was found that osteolysis was also one of the unpleasant outcomes of BMP treatment, resulting in low bone healing efficacy and even increased microfracture [6]. Elaborate gene-modified animal models have confirmed that BMP signaling pathway has a complex effect in bone remodeling, *i.e.*, it promotes osteoblastic differentiation *via* binding with BMP receptor type 1B (BMPR1B) [7], BMP receptor type 1A (BMPR1A) and activin A receptor type 1 (ACVR1) [8,9], whilst it promotes osteoclastic differentiation by osteoblast-osteoclast communication *via* upregulation of *Dkk1* and *Sost* in osteoblasts and subsequent inhibition of WNT signaling in osteoclasts, leading to higher activities of osteoclasts and bone resorption [10,11]. It is known that the canonical (β-catenin-dependent) WNT signaling pathway also plays a critical role in osteogenesis [12], therefore, maintaining the activation of WNT pathways seems a promising approach for efficient application of BMPs in bone regeneration. Considering that BMP pathway can function as either the downstream or upstream of WNT pathway [13], it is necessary to identify an appropriate

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functioning sequence of the two pathways for the best synergistic effects to bone regeneration.

Growth factors are often indispensable for activating signaling pathway, and then successful tissue regeneration, however, the expensiveness and short half-life periods have limited their application. Researchers have developed alternatives with low cost and good biocompatibility to stimulate the expected signaling pathway. Among those, FK506 is an immunosuppressive agent with an increasing clinical application [14], not only has FK506 been proved to suppress inflammation which hinders bone remodeling [15,16], also been reported to activate BMP/Smad signaling and promote osteogenic differentiation through segregating the FK506-binding protein 12 (FKBP12) from binding to and inactivating the BMP receptors [17,18]. Meanwhile, 6–bromo-indirubin-3′-oxime (BIO), a specific inhibitor of GSK3β, leads to promote the stabilization and the subsequent nuclear translocation of β-catenin by decreasing the phosphorylation of β-catenin [19]. Thus, BIO can activate WNT signaling and promotes the osteogenic differentiation of human mesenchymal stem cells [19,20]. Therefore, in this work, FK506 and BIO will be loaded on scaffolds and employed as inexpensive alternatives of BMPs and WNTs, to activate respective signaling.

Numerous scaffolds, including natural polymers, synthetic polymers, and ceramics, have been developed, which can carry drugs attempting to regenerate bone. *Brasenia schreberi* is a rare and precious vegetable, which coat with a gelatinous mucilage [21]. The mucilage coating can protect itself, and has been reported to have antialgal and antibacterial properties [22]. This double-layer structure provides a construction idea for sequential release of drugs. Hydrogels, owing to their good biocompatibility and high efficiency in drug-loaded and release are widely used [23]. Alginate (ALG)-based scaffolds can be prepared with multiple cross-linking methods, and used in various biomedical applications like wound healing and delivery of drugs or other bioactive agents [24,25]. Chitosan (CS), mainly extracted from crustaceans, is the most abundant natural amino polysaccharide. CS hydrogels have wide applications because of their high-water content, which are similar to glycosaminoglycans, the main component of the extracellular matrix (ECM) in natural living tissues [26]. Moreover, ALG and CS hydrogels share advantageous characteristics of low toxicity, abundant resources, low cost, ideal biocompatibility, biodegradability, hydrophilicity, anti-bacterial effect and gel forming property [27,28], ALG hydrogels could fill the bone defect due to their swelling behavior [29], and relatively fast degradation rate, that drug can be released and degraded in a short time in the early stage. CS hydrogels were degradation slowly, that used to drug release for a long time. Thus, ALG hydrogels were designed to form the outer layer, CS hydrogels were used as the inner layer. A two-layered heterogenous hydrogel scaffold mimics the double-layer structure of *Brasenia*, composed of ALG and CS and loaded with FK506 and BIO, respectively, could provide a promising solution to achieve sequential activation of BMP and WNT signaling pathways.

In this study, we prepared a *Brasenia*-inspired heterogeneous hydrogel that the sustained and sequential release of BMP signaling activator and WNT signaling activator was achieved successfully. We evaluated its effect in osteoblastic differentiation and bone formation *via in vitro* and *in vivo* approaches. This novel hydrogel system provides promising alternatives for clinical bone loss treatment with lower costs and higher effects.

In this work, the heterogeneous hydrogels were synthesized as illustrated (Fig. 1). The overall diameter and thickness of the cylindrical heterogeneous hydrogels were 5.0 mm and 2.0 mm, respectively, and the inner CS hydrogels displayed a diameter of 3.0 mm and a thickness of 1.0 mm (Fig. 2A). The morphology and structure of cross section of the heterogeneous hydrogels were characterized by scanning electron microscopy (SEM) imaging, which showed that the outer ALG hydrogels were less porous (Fig. 2B). Nevertheless, studies have reported that the pore size increased after ALG hydrogels are immersed in simulated body fluid, which therefore promoted the growth and the proliferation of cells [24]. Meanwhile, the inner CS hydrogels exhibited a distinctly porous morphology, with a pore diameter ranging from 100.0 μm to 200.0 μm (Fig. 2B), which could facilitate the cellular adhesion, migration and proliferation as well [30,31]. There was no difference in terms of the morphology between heterogeneous hydrogels loaded with or without drugs (Fig. S1 in Supporting information). The stress-strain curve indicated that Young's modulus reached 25.4 kPa, the fracture energy reached 12,407.0 J/m^2, and the compressive strength reached 530.7 kPa at the strain of 99.6% (Fig. 2C), suggesting that the heterogeneous hydrogels could provide structural strength when being applied as a scaffold in tissue engineering. While added the FK506 and BIO, the compressive strength reached 528.1 kPa at the strain of 99.6%, that did not affect the above mechanical indices of the heterogeneous hydrogels (Fig. 2C).

The degradation pattern revealed that as culture time elongated, the weights of the heterogeneous hydrogels got decreased. The degradation rate of the outer ALG hydrogels was initially slow that only 42.1% of ALG got degraded during the first 7 days, but turned rapid from D7 to D10 (Fig. 2D). After 10 days, a complete degradation of ALG was observed. Meanwhile, the inner CS hydrogels started to slowly degrade, and there was still 12.1% CS remaining at D70 (Fig. 2D). This sequential degradation of the ALG/CS heterogeneous hydrogels could provide temporal releasing cues to the loaded drugs, therefore, the drug releasing profiles were examined. It was shown that 44.0% of FK506 loaded within the outer layer released in the first 24 h, with the releasing rate became slower until day 10 when the FK506 within the outer layer released completely, exhibiting a similar pattern to the ALG degradation. As to BIO loaded in the inner layer, a burst release during the first 24 h (13.8%) was observed, which may due to the drug release from the surface of the inner CS hydrogels. Following that, a releasing plateau from D1 to D5 (13.8% to 15.6%), a relatively rapid release (43.9% to 67.5%) from D7 to D10, and a following slow release until D28 (Fig. 2E) were seen. On day 7, the cumulative release of FK506 (in outer ALG hydrogel) reached 91.7%, whereas the cumulative release of BIO (in inner CS hydrogel) was 43.9%, indicating that by generating layered hydrogels with heterogeneous components, we could successfully obtain sustained and sequential release of loaded drugs.

In order to identify appropriate concentrations of FK506 and BIO used in the *in vitro* experiments, CCK8 assay was performed in cultured primary calvarial osteoblasts (preosteoblasts, pre-OBs). Results showed that FK506 concentrations higher than 4.0 μg/mL (Fig. S2A in Supporting information) or BIO concentrations higher than 1.0 μmol/L (Fig. S2B in Supporting information) exhibited toxicity to pre-OBs. These results were in accordance with previous publications that a concentration lower than 8.0 μg/mL of FK506 [18,32], and a concentration lower than 2.0 μmol/L of BIO showed no obvious toxic effects [20,33]. To assess the optimal concentrations of FK506 and BIO on osteogenesis, pre-OBs were treated with different concentrations of FK506 (1.0, 2.0, 4.0 μg/mL) or BIO (0.1, 0.5, 1.0 μmol/L), respectively, ALP and AR staining were performed. The ALP positive areas at D7, as well as the quantitative data of AR staining at D21, showed that 1.0 μmol/L BIO and 4.0 μg/mL FK506 exhibited the most pronounced effect in promoting the osteogenic differentiation of pre-OBs (Figs. S3A-C in Supporting information), and therefore were used in subsequent experiments.

Previous studies have shown that elongated or excessive activation of BMP or WNT signaling pathway may lead to undesired outcomes like ectopic bone formation [13], therefore, FK506 and BIO were used no more than 14 days in this work. Moreover, it is known that BMP and WNT signaling pathway both play important roles in bone formation and the relationship between them is com-

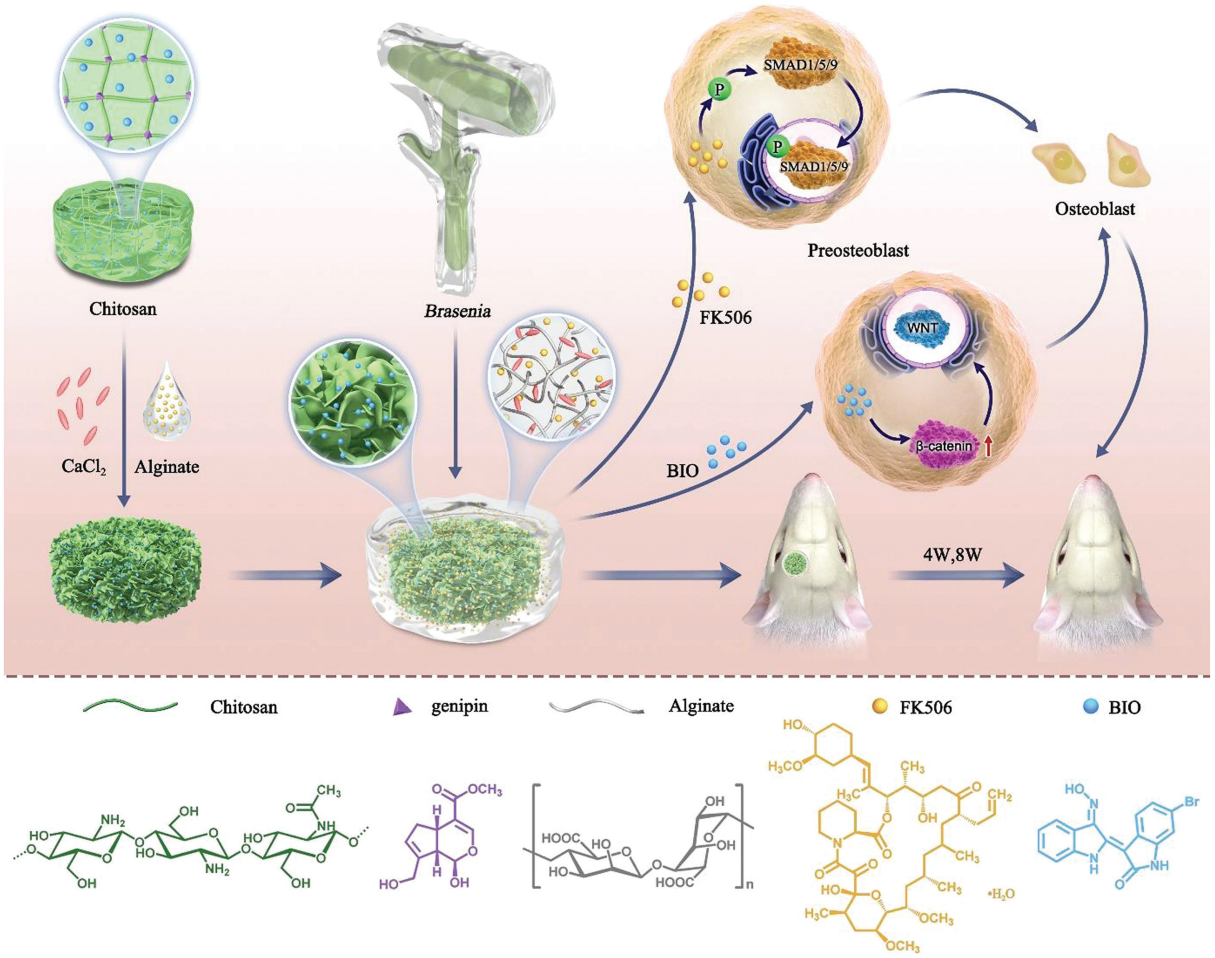

Fig. 1. Schematic illustration of the preparation of drug-loaded sodium alginate (ALG)/chitosan (CS) heterogeneous hydrogel system and its role in promoting bone regeneration.

plicated, and the bone regeneration effect largely dependent on the differentiation status of the cells [13]. In order to identify the advantageous releasing sequence of FK506 and BIO, pre-OBs were treated with osteogenic differentiation medium, together with one drug in the first 7 days, that consistent with the degradation of the outer layer ALG hydrogel. Then replaced by the other drug for the next 14 days, and followed by AR staining at D21. AR staining and its quantitative data showed that the sequential treatment of 4.0 μg/mL FK506 for the first 7 days and 1.0 μmol/L BIO for the following 14 days achieved the highest amount of calcium deposition, although no significant difference was found between this combinational group with other groups (Figs. S3D and E in Supporting information). Therefore, FK506 with a concentration of 4.0 μg/mL and BIO with a concentration of 1.0 μmol/L were used for following experiments.

To further explore the role of the drug-loaded heterogeneous hydrogels on osteogenic differentiation, pre-OBs were treated with the supernatants of the drug-loaded heterogeneous hydrogels and examined by ALP and AR staining. As to ALP staining, no significant difference in ALP-positive areas was found among the blank, control and B-B groups or among the F-F, B-F and F-B groups, while the latter three groups exhibited significantly larger ALP positive areas than the former three groups (Figs. 3A and B). These results suggested that unloaded or BIO-only-loaded heterogeneous hydrogels barely induced ALP activity, while the heterogeneous hydrogels loaded with FK506 alone or a combination of FK506 and BIO could significantly induce ALP activity *in vitro*.

As to AR staining, the F-B group possessed more calcium deposition compared with all other groups, amongst which no difference was detected (Figs. 3A and C). Quantitative RT-PCR results further confirmed that the addition of the supernatants from the heterogeneous hydrogels could significantly upregulate the expressions of some osteogenic genes. *Runx2* is an early marker for osteogenic differentiation. *Sp7* and *Col1α1* function as downstream effectors of *Runx2*. *Ocn* is related to terminal differentiation of osteoblasts [17]. Our results showed that compared with the control group, F-B group significantly induced *Runx2* expression at D3 (Fig. 3D), and all experimental groups increased *Sp7* expression at D7 and D14 (Fig. 3E), and some groups enhanced *Col1α1* expression at D7 (control, B-B and F-B groups) and D14 (B-B and F-B groups) (Fig. 3F), and B-B, F-F and F-B groups elevated *Ocn* expression at D14, moreover, F-B group significantly higher than control group (Fig. 3G). These results confirmed that the sequential release of FK506 and BIO *via* the heterogeneous hydrogel system effectively improved the osteogenic differentiation of pre-OBs. To be noted, the F-B group displayed higher expression of all mentioned osteogenic genes stably at all these time points, therefore, this kind of combination was used for following experiments as the most promising one to promote new bone formation.

As the heterogenous hydrogel drug delivery system will be applied *in vivo* without cells, we hypothesized that the drug released from the heterogenous hydrogels could promote the migration of bone marrow mesenchymal stem cells (BMSCs). To test this hypothesis, we performed transwell migration assay using the supernatants from heterogenous hydrogels within 24 h. The results

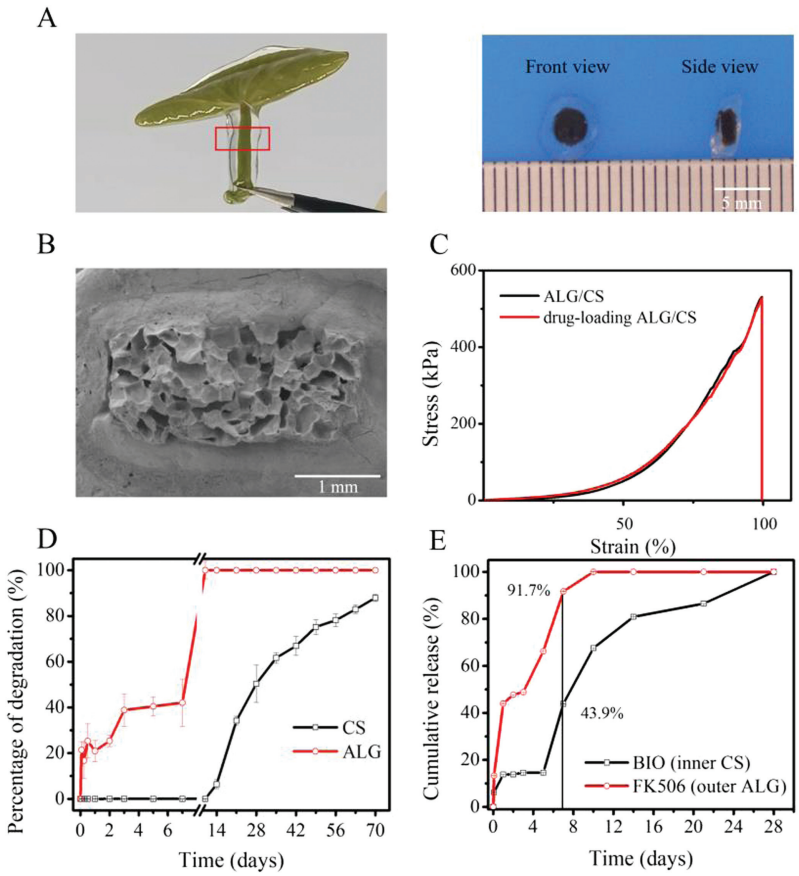

Fig. 2. Preparation and characterization of the ALG/CS heterogeneous hydrogels. (A) *Brasenia* figure (left), front and side views of the heterogeneous hydrogels (right). (B) SEM image of the drug-loaded heterogeneous hydrogels. (C) Mechanical characterization of the heterogeneous hydrogels. (D) Degradation behavior of the heterogeneous hydrogels. (E) Release profile of the inner and outer layer drugs from ALG/CS heterogeneous hydrogels. Data are represented as means ± SD from three experiments.

showed that the supernatants from all the experimental groups could significantly promote the migration of BMSCs, while F-B group could further promote the migration of BMSCs compared with other experimental groups (Fig. S4 in Supporting information).

To further examine the role of the drug-loaded heterogeneous hydrogels in activating the BMP and WNT signaling pathways, pre-OBs were treated with the supernatants collected at different time points from the drug-loaded heterogeneous hydrogels, and harvested for western blot assay. Results showed that with the addition of supernatants collected at D1–3 and supernatants collected at D4–7, only the F-B group significantly elevated the P-SMAD1/5/9 level compared to the control group (Figs. S5A and C in Supporting information). Instead, when the supernatants collected at D8–14, the P-SMAD1/5/9 levels were similar among all the groups (Fig. S5E in Supporting information). These results not only further confirmed our drug release profile in Fig. 2E that 48.8% and 42.8% drugs got released from the outer layer of the heterogeneous hydrogel at D1–3 and D4–7, respectively, but also proved that the release of FK506 successfully activated the BMP/Smad signaling pathway.

As to the WNT/β-catenin pathway, the results showed that only the supernatants collected at D4–7 from the B-B group and collected at D8–14 from the F-B group significantly increased the level of β-catenin compared to the control group (Figs. S5D and F in Supporting information). These results showed that the activation of WNT/β-catenin pathway required a certain amount of BIO, which were acquired from the D4–7 release of B-B group and the D8–14 release of F-B group as shown in Fig. 2E that 36.0% and 37.1% of BIO got released, and activated the WNT/β-catenin signaling pathway.

To further evaluate the potential clinical application of the drug-loaded heterogeneous hydrogels, we firstly employed subcutaneous implantation *in vivo*. All rats were maintained and utilized according to the ethical regulations of Jilin University. The animal protocols (No. SY202011006) were approved by the Laboratory Animal Use and Care Committee at Jilin University. The results showed that implanting the system subcutaneously did not cause local inflammation or necrosis of the tissues (Fig. S6 in Supporting information). We also implanted the hydrogels in a rat calvarial defect model, a standard *in vivo* model for bone regeneration evaluation. H&E staining of important organs, *i.e.* heart, liver, spleen, kidney and lung, showed no toxic reaction to drug-loaded heterogeneous hydrogels (Fig. S7 in Supporting information). After confirming the good biocompatibility of the drug-loaded heterogeneous hydrogels, we performed micro-CT scanning as well as the histomorphometrical analyses *via* H&E staining and Masson's trichrome staining to quantify the neo-bone volume and quality. The results clearly showed that only a small amount of new bone formed at the edges of the bone defect which was connected to the host bones in the control group and B-B group, as indicated by reversal lines (Fig. S8A in Supporting information and Fig. 4A (first line, black arrows)). Quantitative data showed that 4 weeks after surgery, the volumes of newly formed bone were 21.59% and 38.63% of the defect area in the control group and B-B group (Figs. S8A and B in Supporting information), respectively, while after 8 weeks post-surgery, they took 33.24% and 51.61%, respectively (Figs. 4A and B). Meanwhile, more bone formation was observed at the edges

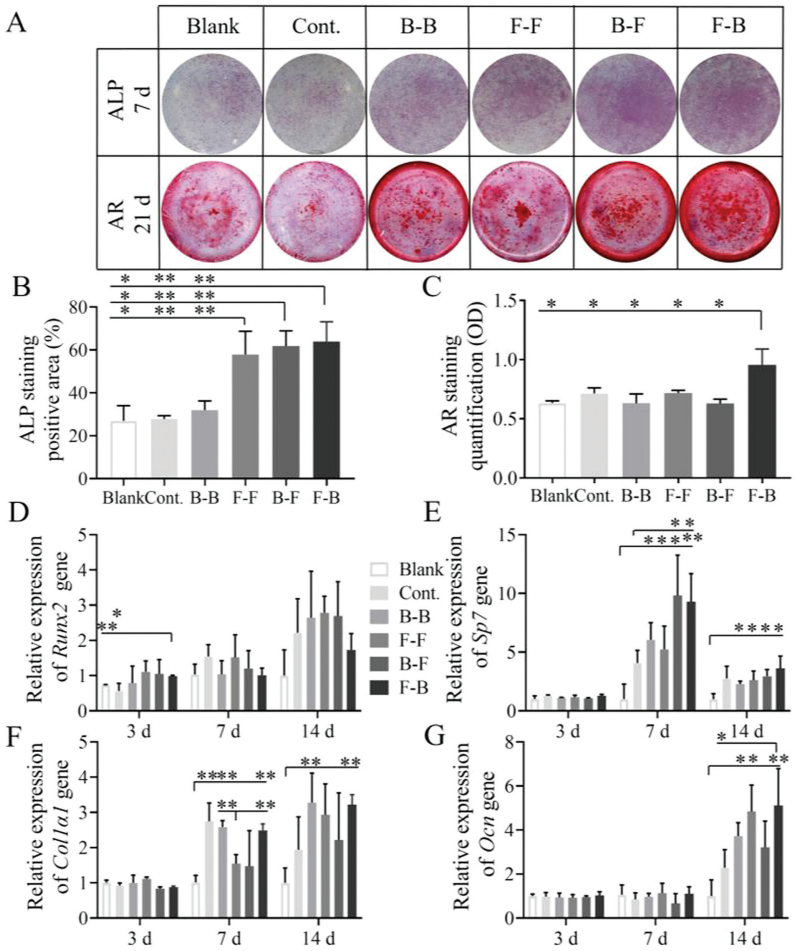

Fig. 3. The effects of drug-loaded heterogeneous hydrogels on the osteoblastic differentiation of mouse calvarial pre-osteoblasts (pre-OBs). (A) Representative images of ALP staining and AR staining. (B and C) Quantification of ALP staining and AR staining. All groups were treated with osteogenic medium. Abbreviations: Blank: no hydrogels or drugs. Cont.: Control ALG/CS heterogeneous hydrogels without FK506 or BIO. B-B: ALG/CS heterogeneous hydrogels with BIO (WNT signaling activator) loaded in both ALG and CS hydrogels. F-F: ALG/CS heterogeneous hydrogels with FK506 (BMP signaling activator) loaded in both ALG and CS hydrogels. B-F: ALG/CS heterogeneous hydrogels with BIO loaded in ALG hydrogels and FK506 loaded in CS hydrogels. F-B: ALG/CS heterogeneous hydrogels with FK506 loaded in ALG hydrogels and BIO loaded in CS hydrogels. Data are represented as means ± SD from three experiments. (D-G) Gene expressions of *Runx2* (D), *Sp7* (E), *Col1α1* (F), and *Ocn* (G) were analyzed by quantitative RT-PCR assays in pre-OBs after 3, 7 and 14 days cultured with osteogenic medium. * $P < 0.05$; ** $P < 0.01$.

and the central areas of the defect in the F-F group (52.83% at 4 weeks and 73.99% at 8 weeks) and B-F group (54.52% at 4 weeks and 73.81% at 8 weeks), and quantitative data showed that significantly larger amount of bone formed compared with the control group and B-B group. It is noteworthy that the F-B group exhibited not only the largest amount of new bone formation (86.96%), but also significantly higher levels of trabecular number (Tb. N) (Fig. 4C), bone area (BA) (Fig. 4E), new bone length (Fig. 4F) as shown by further quantitative analyses of the new bone after 8 weeks, indicating that F-B hydrogel have the strongest capability of inducing bone regeneration (Figs. 4A–G). In Masson staining, mature bones are stained bright red, whereas immature bones are stained blue. Masson staining in Fig. 4A showed that in control group, the newly formed bones were stained blue, indicating immature bones. In B-F group, immature bones (stained in blue) predominated the majority of newly formed bones. While, in B-B, F-F, and F-B groups, the newly formed bones were mainly mature bones (stained in red). These results suggested that F-B group not only can effectively induce bone regeneration, but also accelerate the maturation of the newly formed bones.

In order to verify our drug-loaded heterogeneous hydrogels could also be applied in bone defects of cancellous bones, we performed a rat femoral bone defect model as well. The results were in accord with the results of calvarial defect model (Figs. S9 and S10 in Supporting information), demonstrating that all experimental groups exhibited bone regeneration, with the new bone formation the most significant in F-B group.

In our work, we found that the F-B group supported most bone regeneration *in vivo* than the B-B, F-F, and B-F groups. Firstly, this indicated that a combinational activation of BMP and WNT signaling pathway possessed greater potential than the activation of BMP or WNT pathway alone. Moreover, these results implied that pre-OB osteogenic differentiation depended on not only the concentrations of FK506 and BIO, but also the functioning sequence of the two drugs. Early release of FK506 to activate the BMP/Smad pathway followed by the activation of WNT/β-catenin pathway *via* BIO exhibited a most superior effect.

Previous studies have proved that the functions and relationship of BMP and WNT pathways depend on the differentiation stages of cells [13]. As to undifferentiation mesenchymal precursor cells, it was shown that WNT/β-catenin pathway dominates the directed differentiation of the cells towards osteoblast progenitors, afterwards, it functions to promote cellular proliferation and maintain the precursor status, while BMP signals stimulate the osteoprogenitors to become mature osteoblasts [34]. In the meantime, a recent study revealed that in *Bmpr1a*-deletion mice, multiple WNT

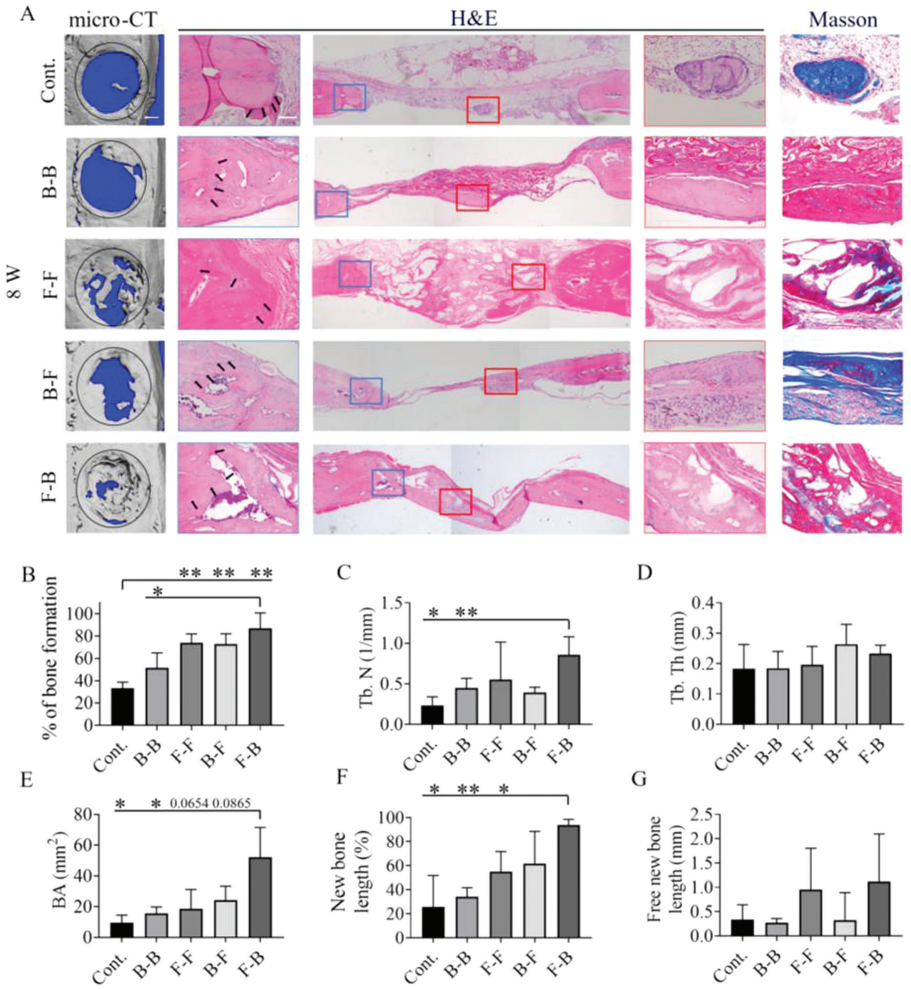

Fig. 4. Bone regeneration of rat calvarial bone defects 8 weeks post-implantation. (A) Micro-computed tomography (micro-CT) reconstruction images, H&E staining and Masson's trichrome staining of the calvarial bone defects. The black circles indicate the original defect. Scale bar in micro-CT images = 1 mm. Black arrows in histological sections indicate reversal lines. Scale bar = 500 μm and 100 μm for low and high magnifications, respectively. (B–E) Quantifications of new bone formation by micro-CT. Tb. N, trabecular number; Tb. Th, trabecular thickness; BA, bone area. (F and G) Histomorphometrical analyses of the regenerated bone in calvarial defects. Results are shown as mean ± SD ($n = 3$). * $P < 0.05$; ** $P < 0.01$.

related genes were markedly downregulated, indicating that the BMP signaling could function as an upstream of WNT signaling. Meanwhile, the overexpression of *Wnt7b* rescued the phenotype of excessive trabecular bone formation in *Bmpr1a*-deletion mice, implying that the WNT signaling pathway could exhibit somewhat compensational role to BMP signaling [35]. However, when BMP and WNT pathways were activated simultaneously, it was shown that BMPs antagonized the WNT signaling by promoting the interaction between *Smad1* and *Dvl-1* that restricted β-catenin nuclear translocation and activation in osteoblast progenitors [36]. Taken together, the sequential functions and the relationship between the BMP remained controversial and required further study.

Considering our data that the F-B group exhibited higher but not significant levels of the osteogenic genes and proteins *in vitro* studies, but distinctly more bone formation *in vivo* than the F-F, B-B, and B-F groups, we speculated that the difference could be caused by following two reasons. Firstly, as previous studies have confirmed that activated BMP signaling have beneficial effects in inducing site-directed cell homing [37,38], we presumed that the early release of FK506 in the bone defect recruited more osteogenic precursors, laying the foundation for following bone formation. While in our *in vitro* studies, since only supernatants from the heterogeneous hydrogels were used and the cell migration was not studied, the cell-homing effects were not explored. Secondly, bone defect reparation includes not only osteoblast-directed bone formation but also osteoclast-directed bone resorption. It was shown that in differentiated osteoblasts, activation of WNT/β-catenin promoted the ability of differentiated osteoblasts to inhibit osteoclast differentiation *via Opg*, a known direct target of β-catenin [39]. Therefore, a latter activation of WNT/β-catenin could suppress the osteoclastogenesis *via* the differentiated osteoblasts which were induced by the early activation the BMP pathway in the F-B group, leading to most bone formation *in vivo*. Therefore, in order to clarify the possible mechanisms underlying these results, further studies including the influence of cell migration, cell proliferation and osteoclastogenesis of the heterogeneous will be necessary.

Our design is inspired by the *Brasenia*, combined with ALG hydrogel and CS hydrogel to form a heterogeneous hydrogel. The study demonstrates that heterogeneous hydrogel not only provide sufficient mechanical strength and good biosafety, but also achieved a sustained and sequentially release FK506 and BIO, which efficiently repair bone defect without multiple administration. Therefore, our results suggest that heterogeneous hydrogel loaded with FK506 and BIO has great potential for future clinical application. Moreover, we confirmed that a sequential activation of

BMP and WNT signaling possessed highest potential in inducing bone regeneration. Further work will be focused on the exploration of the underlying molecular mechanism.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.cclet.2022.107965.

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