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Sustained knock down of PPARγ and bFGF presentation in collagen hydrogels promote MSC osteogenesis

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Abstract: Collagen hydrogels were considered as favourable scaffolding for tissue engineering. It was demonstrated that cytokines and siRNAs could be efficiently retained by collagen hydrogels for controlled release thereby enhancing their bioactivities. Basic fibroblast growth factor (bFGF) was a stimulator for osteogenic differentiation of mesenchymal stem cells (MSC), and PPARγ was a key regulator in MSC osteogenic differentiation. However, whether bFGF and PPARγ could play synergetic roles within a 3D matrix to promote MSC osteogenic differentiation was unknown. In the study, bFGF and PPARγ targeting siRNAs were incorporated into collagen hydrogels for MSC cultivation. Their optimal concentrations in collagen hydrogels were determined. The capacity of bFGF/siRNA-carrying hydrogels in supporting osteogenic differentiation of MSCs was systematically evaluated with multimodality of methods, including flow cytometry, quantitative real-time PCR, Western Blotting, as well as ALP activity and calcium content determination. We demonstrated in 3D collagen hydrogel that both bFGF and siRNA molecules were efficiently retained, strengthening their effects on the incorporated MSCs. Osteogenic analysis demonstrated that the *in-situ* forming hydrogels carrying bFGF and siRNAs potently promoted osteogenic differentiation of incorporated MSCs, significantly superior to pure collagen and bFGF-carrying collagen. Thus, collagen hydrogels functionalized with bFGF and PPARγ targeting siRNAs may be promising in bone tissue engineering.

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1 Introduction

In modern times, bone fractures which lead to lots of serious bone defects occur frequently.. Meanwhile, severe bone disorders are also increasingly prevalent with the greying of society [1,2]. For serious bone defects, it is usually difficult to heal through endogenous regenerative mechanisms, especially for aged patients. Extrinsic regenerative strategies, therefore, attract more and more attention from clinicians and investigators [3,4].

In recent years, development of bone tissue engineering demonstrated a promising perspective for the treatment of severe bone defects and was extensively studied [5,6]. Biomaterials, which may support the survival and guide osteogenic differentiation of seeding cells [7], were confirmed to play important roles in bone tissue engineering [8]. In the past years, several biomaterials were investigated as supporting matrix for bone tissue engineering, such as alginate and nanofibers [9-11]. Collagen, a natural extracellular matrix, has a good biocompatibility for stem cells survival and could form hydrogels in situ to retain cells and thus was widely used in tissue engineering, including bone tissue engineering [12-14]. For enhancing the therapeutic effects, it is essential to guarantee the osteogenic differentiation of seeding cells within scaffolds [14]. However, pure collagen itself was limited in guiding osteogenic differentiation of stem cells. To enhance the osteogenic bioactivity of the collagen matrix, some investigators introduced bioactive growth factors matrix [15]. bFGF, which was confirmed as a stimulator to promote osteogenesis [14], was introduced into collagen hydrogels to enhance osteogenesis of embedded mesenchymal stem cells

(MSC) for bone tissue engineering [14,16]. It was found that incorporated bFGF could be efficiently retained within hydrogels to support osteogenic differentiation of surrounding MSCs, providing a promising way to functionalize the scaffold materials in bone tissue engineering [14].

Though appropriate growth factors were useful in enhancing the bioactivity of biomaterials for stem cells osteogenic differentiation, single cytokine was usually insufficient to achieve a satisfied effect as confirmed in previous reports [17]. Therefore, some other strategies, which may play synergetic roles with growth factors in regulating MSC differentiation, would be required in bone tissue engineering. Peroxisome proliferator-activated receptor gamma (PPARγ) was confirmed to be an important regulator in MSC osteogenic differentiation [2,18]. It has been demonstrated that down-regulation of PPARγ could significantly promote bone regeneration [18]. In a report by Lee *et al.* [2], suppression of PPARγ through RNA interference efficiently enhanced MSC osteogenic differentiation induced by osteogenic medium, suggesting that suppressing PPARγ by siRNA may be a potent enhancer for MSC osteogenic differentiation.

In addition to controlling cytokine release, collagen hydrogel was also confirmed as able to control siRNA release [19]. More importantly, it was demonstrated that collagen hydrogels carrying siRNAs could suppress the target gene of incorporated cells more efficiently and for a longer time. We hypothesized that incorporating osteogenic cytokine together with PPARγ-targeting siRNA in collagen hydrogel may produce a synergetic role in guiding osteogenic differentiation of embeded stem cells and thus resulted in a favorable matrix for bone tissue engineering. Accordingly, we designed the study and aimed to demonstrate 1) whether regulating PPARγ (a key osteogeneic regulator) together with bFGF could synergistically promote MSC osteogeneic differentiation compared with a single osteogenic agent; and 2) whether embedding bFGF together with PPARγ-targeting siRNA in collagen hydrogel could endue the scaffold with osteogenesis-promoting bioactivity. In the study, PPARγ-targeting siRNA together with bFGF was incorporated into collagen hydrogels for cultivation and osteogenic differentiation of MSCs. The concentration of siRNAs and bFGF were optimized. The release profiles of bFGF and siRNAs were assessed. The capacity of the bFGF/siRNAs containing hydrogels in guiding MSC osteogenic differentaition were systematically evaluated.

2 Materials and methods

2.1 Isolation, cultivation and characterization of human mesenchymal stem cells

Mesenchymal stem cells were isolated from human adipose tissues according to previous reports with some modifications [7,20]. Briefly, adipose tissues were obtained from raw human lipoaspirates and extensively washed by sterile phosphate buffer solution to remove contaminating debris and red blood cells. Then, adipose tissues were cut into < 1 mm^3 pieces and digested with 0.1% collagenase I (Sigma) supplemented with 0.05% trypsin in serum-free medium (α-minimum essential medium, α-MEM, Gibco). After 30 min digestion at 37°C with gentle agitation, the enzymes were inactivated with an equal volume of α-MEM containing 10% fetal bovine serum (FBS). The digested solution was filtrated through 80 μm meshes and the filtrate was centrifuged for 5 min at 800 g. The cellular pellet was resuspended in fresh αMEM/10% FBS. Cells were counted with haemocytometer and plated onto tissue culture plates in α-MEM/10% FBS. Cells were incubated at 37°C, 5% CO_2 condition. When they reached 90% confluence, cells were collected by 0.25% trypsin digestion and passaged at 1:3. After three passages, flowcytometry was performed to analyze the cell immunophenotype after staining by FITC or PE conjugated-antibodies according to the previous report [21].

2.2 Preparation of collagen hydrogels

Collagen hydrogels were prepared as previously described [19] with slight modification. Briefly, collagen solution (Rat tail Col I; BD Science) was mixed with α-MEM (10×Gibco) and distilled water, to obtain an appropriate nutritional environment for the culture of cells. 1N NaOH was added to neutralize the mixture to pH 7.4. The final concentration of collagen was determined to be about 2 mg/mL. When placed in 37°C, 5% CO_2 condition, the solution would solidify into a hydrogel matrix in minutes.

2.3 bFGF and siRNA Release

To incorporate the bFGF or siRNA (targeting PPARγ) into collagen hydrogel, bFGF or siRNA was added to distilled water before the preparation of collagen

hydrogels as described above. For measurement of bFGF release, different concentrations of human bFGF (0.5, 1 and 2.5 μg) were incorporated into collagen hydrogels. Before hydrogel formation, collagen solution containing bFGF was poured into 24-well plates and allowed to solidify. Then, culture medium was added and plates were placed in an incubator at 37°C. At different time points, the supernatant was collected and the bFGF was determined by an enzyme-linked immunosorbent assay (ELISA) kit (Peprotech) according to the manufacturer's instructions.

For siRNA release studies, collagen solution containing siRNA was poured onto transwell membranes with 0.4 μm pore size. After gelatination, the transwell membranes were placed into the wells of a 24-well plate and PBS was added to each well. At different time points, the PBS was collected and the siRNA content was measured using the RiboGreen RNA quantitation reagent according to the previous report [19].

2.4 Cell proliferation and survival assay

For assaying the effect of bFGF on cell proliferation within collagen hydrogel, 1×10^4 MSCs were incorporated into 500 μL hydrogels and poured onto 24-well plates. At different time points during the cultivation, cells were collected by digesting collagen with type I collagenase (10 mg/mL). Cells were quantified using an MTT (3-(4,5-dimethylthiazol-2-yl)-5- (3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay kit assay kit (Promega) according to the previous report [14] and the manufacturer's instruction. The 490 nm absorbance was measured using a spectrophotometer. To assay cell viability in siRNA containing hydrogel, MSCs were incorporated into siRNA-containing hydrogels and incubated in 37°C, 5% CO_2 condition for 24 h. Then, the collagen was stained with Live/Dead assay kit (Invitrogen) according to the manufacturer's instruction. Dead cells were counted under fluorescencent microscope. The cell viabilities were expressed as viable cells/total cells.

2.5 Western blotting

Cell samples were collected by digesting collagen hydrogel with collagenase I. Then, the collected cells were lysed in Laemmli Sample Buffer (Bio-Rad). Ultrasound treatment was performed to facilitate cell lysis. Protein concentrations were determined using the BCA™ Protein Assay Kit (Thermo Scientific). Then, 60-80 μg of proteins were loaded on a 15% sodium dodecyl sulfateepolyacrylamide gel for electrophoresis. After that, proteins were transferred to a PVDF membrane (Roche). The membrances were blocked with 5% nonfat dried milk (in TBST) for 2 h and then were incubated with primary antibodies at 4°C overnight. The membranes were washed with TBST three times to remove unconjugated antibodies and then incubated with horseradish peroxidase-conjugated secondary antibodies at room temperature for 1 h. Enhanced chemiluminescence reagent (Applygen) was employed to detect the protein bands. GAPDH was used as an internal standard. The signal intensities were normalized to corresponding internal standards and expressed as the percentages of control.

2.6 Quantitative real-time PCR (QPCR)

At different time points, cells were collected from collagen hydrogel by collagenase digestion and centrifugation. After lysis with trisol, total RNA was extracted using RNeasy Mini Kit (Qiagen Ltd.). Then, Superscript kit (Invitrogen) was used for reverse transcription (RNA to cDNA) with random hexamers. Sensimix Plus SYBR master mix (Quantace) and a spectrofluorometric thermal cycler (Rotor-Gene 3000) were employed for the following QPCR experiment. The gene expression was normalized to the folds of its control. Triplicate samples were tested.

2.7 Fluorescence-activated cell sorting analysis

At predetermined time points, cells were collected by digesting collagen with collagenase I as described above. The harvested cells were washed with PBS and fixed with 2% paraformaldehyde. Then, cells were treated with 0.2% Triton X-100. After blocking with serum albumin (BSA), anti- OPN antibodies were added and incubated with cell samples for 1h at room temperature. The redundant primary antibodies were removed by washing with PBS and then, FITC-conjugated secondary antibodies were added and incubated with cell samples for another 1 h. The cell samples were washed again to remove unconjugated secondary antibodies. OPN-positive cells were counted using flow cytometry system (BD Biosciences).

2.8 ALP activity and calcium content determination

At predetermined time points, samples were collected in cryovials and snap-frozen in liquid nitrogen, which were then stored at -80°C. As previously described [22], all frozen samples were pulverized in liquid nitrogen. To assess ALP activity, samples were treated according to the previous report [22]. Briefly, pulverized sample powders were lysed in 0.2% Triton X-100 using two freeze–thaw cycles. Then, 20 µL lysate was added into 80 µL of 1.5 M 2-amino-2-methyl-1-propanol (AMP; Sigma) buffer (pH 10.3) supplemented with 5.0 mM p-nitrophenol phosphate substrate (Sigma) and then the plate was measured at 405 nm with a spectrophotometer. Serial dilutions of p-nitrophenol (Sigma) were made in Triton solution for plotting standard curve. An OCPC (orthocresolphthalein complex one) method was used to quantify calcium according to the previous report too [3,22]. DNA contents were measured using the PicoGreen kit according to manufacturer's instruction. Both ALP activity and calciumcontents were normalized to DNA contents as previously reported [22].

2.9 Statistical analysis

All data were expressed as mean±standard deviation (SD). SPSS 15.0 software was used for statistical analysis. Analysis of variance was performed for comparison between different groups followed by Tukey's *post hoc* tests. A P value < 0.05 was considered as a difference of statistical significance, and a P value < 0.01 considered as a difference of notably statistical significance.

3 Results

3.1 bFGF release from hydrogel matrix

Different quantities (0.5 µg, 1.0 µg and 2.5 µg) of bFGF was incorporated into collagen hydrogels, and poured into 24-well plates in culture medium. To plot the release profile, bFGF in medium was measured with Elisa at different time points until one month. As shown in Figure 1A, incorporated bFGF was gradually released from collagen hydrogels and demonstrated a stepwise pattern: an initial rapid and steady release within the first 7 days and then sustained a steady release for up to 30 days. Within the first 10 days, about 0.2-0.3 µg bFGF was released from 0.5 µg bFGF-containing hydrogels, 0.5-0.6 µg was released from 1.0 µg bFGF-containing hydrogels and 0.9-1.0 µg was released from 2.5 µg bFGF-containing hydrogels, respectively. At 30 days, about 70-80% (0.5 µg), 60-70% (1.0 µg) and 50-60% (2.5 µg) bFGF was released from hydrogels. The results suggested that lots of bFGF was still retained into collagen hydrogels after 10 days, which may exert persistent effects on incorporated cells.

3.2 Effects of bFGF containing hydrogels on MSC proliferation

To determine the influence of bFGF containing hydrogels on MSC proliferation, MSCs were incorporated into collagen hydrogels together with different amounts of bFGF, cell quantities were determined in 3 day cultures. As shown in Figure 1B, absorbance at 490 nm (positively related with cell numbers) was comparable among different groups

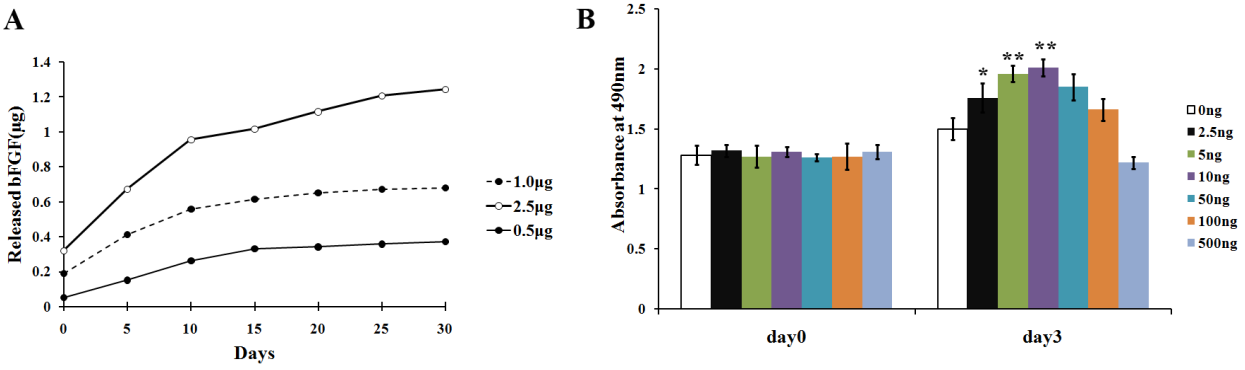

Figure 1. Release of bFGF from collagen hydrogel and effect on MSC proliferation. A, different amounts of bFGF were incorporated into collagen hydrogel. With time, the incorporated bFGF was released in a stepwise pattern: an initial rapid and steady release within the first 7 days and then further sustained and steady release up to 30 days. B, The effect of bFGF in collagen hydrogel on the incorporated MSCs was dose-dependent: benifical for MSC proliferation at low doses, while inhibitory at high dose. The optimal dose was determined as 10 ng (n = 4, *P < 0.05 compared with control; **P < 0.01 compared with control).

(hydrogels containing 0, 2.5, 5, 10, 50, 100, and 500 ng) at day 0, indicating that equal numbers of MSCs were incorporated into hydrogels of different groups. At day 3, absorbance increased with the concentration of bFGF (< 10 ng), the peak absorbance was achieved in 10 ng (bFGF) group. However, when incorporated bFGF was more than 10 ng , the absorbance decreased with the bFGF amount, indicating that excess bFGF would adversely influence MSC proliferation. Collectively, 10 ng bFGF was chosen in the following experiment.

3.3 Optimation of siRNA concentration in collagen hydrogel

To determine the optimal siRNA concentration for MSC survival and gene silencing, MSC viability and PPARγ expression were evaluated after transfection with different amount of siRNAs. As shown in Figure 2A, Live/Dead staining showed that MSC viability was not significantly influenced by siRNAs below 200 μM. Western blotting demonstrated that PPARγ levels decreased with the concentration of siRNAs, and when the concentration was up to 100 μM, about 70% PPARγ expression was silenced compared with control. With the siRNA concentration more than 100 μM, no further silencing of PPARγ was observed. Collectively, 200 μM was chosen for preparation of siRNA-containing hydrogel to ensure the efficient gene silencing.

3.4 siRNA release from hydrogels and sustained PPARγ silencing of incorporated MSCs

siRNAs were incorporated into collagen hydrogels and released siRNAs were detected at predetermined time

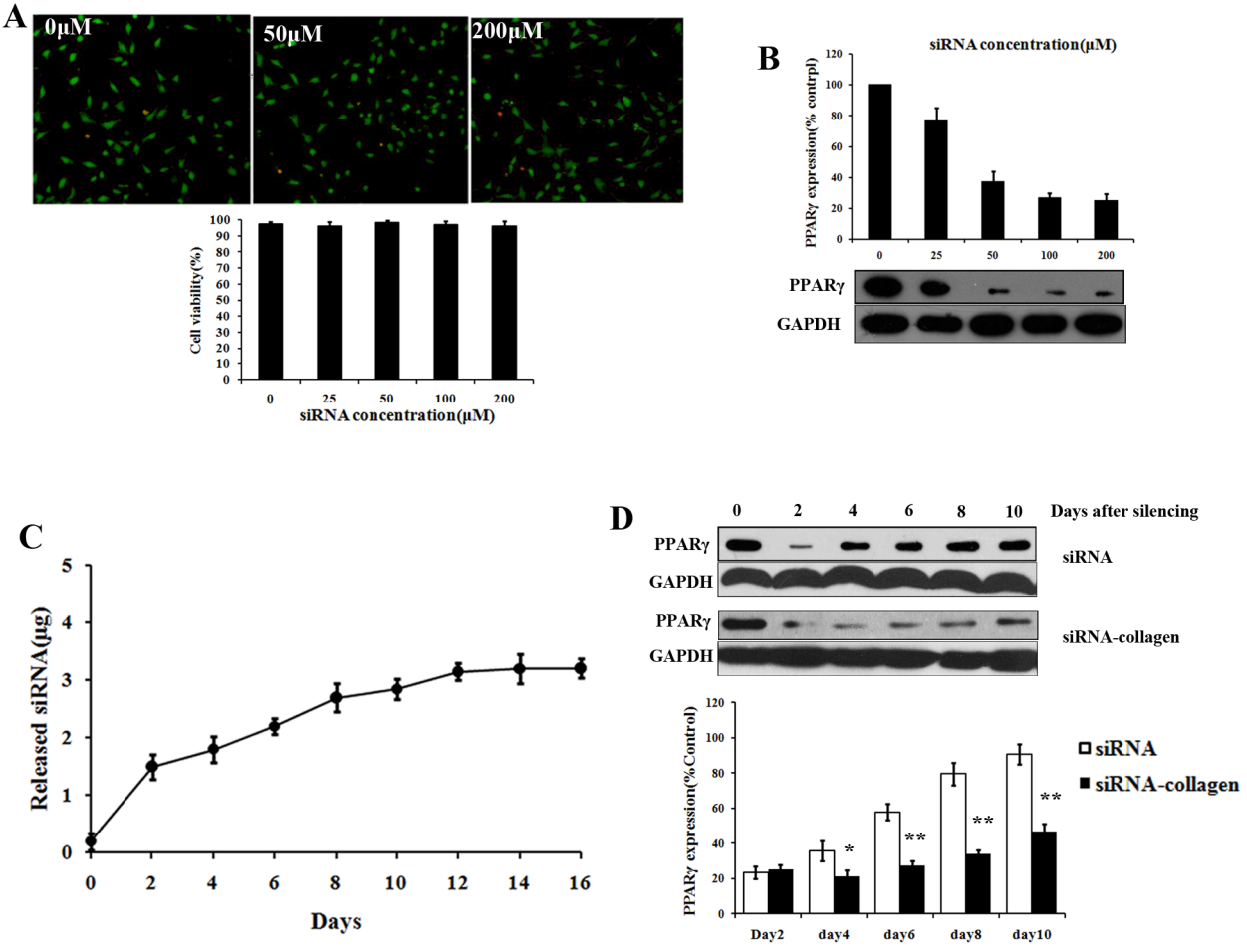

Figure 2. Optimization of incorporated siRNAs into collagen hydrogel. A, Live/dead staining showed that no significant adverse effect of PPARγ-targeting siRNAs was observed on MSC survival up to 200 μM; B, at 100 μM or higher concentration, PPARγ expression was efficiently silenced; C, Rentation and controlled release of siRNAs from collagen hydrogel; D, Compared with conventional method, the silencing of target gene by siRNA-carrying collagen hydrogel was more effective and lasted longer tine (n = 4, **P < 0.01 compared with control).

points until 16 days. As shown in Figure 2C, siRNAs were gradually released from collagen hydrogels with time like bFGF. At 16 days, about 30-40% siRNAs were released from collagen hydrogels, indicating that there were still lots of siRNA restained in hydrogels which may produce sustained effects on incorporated cells.

When MSCs were incorporated into hydrogels with siRNAs, the expression of PPARγ was determined by western blotting. Both free siRNAs and hydrogel-contained siRNAs efficiently suppressed PPARγ expression at day 2 (about 20-30% of control, Figure 2D). In the free siRNAs transfected group, PPARγ levels gradually recovered with time. At one week after transfection, the expression of PPARγ recovered to about 60% of control, indicating that free siRNAs may only efficiently silence the targeted gene within one week. While in siRNA containing hydrogels, the PPARγ level was silenced by about 30% compared with control at one week. Even at ten days, about 50% PPARγ expression was still silenced. The results indicated that the PPARγ silencing was efficiently maintained for longer time in siRNA containing hydrogels.

3.5 Flowcytometry analysis of osteogenic markers

At day 7 and day 14, cells incorporated into hydrogels were collected and immunostained with anti-OPN antibodies. Flowcytometry analysis showed that 4.6% OPN positive cells were detected in havested cells from bFGF-containing hydrogel, significantly higher than those from pure hydrogel (1.2%, $P < 0.01$, $n = 4$), but significantly lower than those from bFGF/siRNA-containing hydrogel (7.2%, $P < 0.01$, $n = 4$) (Figure 3A and B). While at day 14, the OPN-positive cells from pure hydrogel, bFGF-containing hydrogel and bFGF/siRNA-containing hydrogel were 2.1%, 18.4% and 27.7%, respectively.

3.6 Osteogenic gene expression

At day 0, 7 and 14 days after incorporation, MSCs were harvested and osteogenic genes (ALP, OPN, BSP and OCN) were detected by RT-PCR. Compared with control,

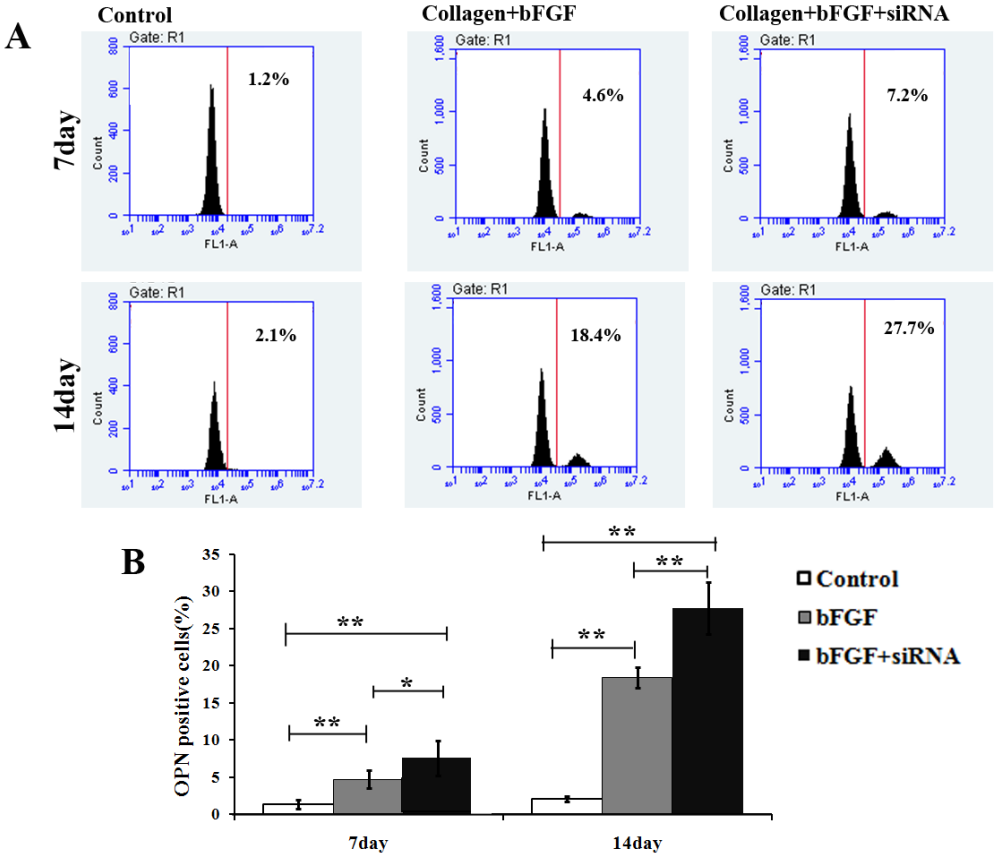

Figure 3. Flowcytometry of OPN positive cells during MSC differentiation. A, After 7days and 14 days differentiation, the most OPN-positive cells were detected in bFGF/siRNA-carrying collagen hydrogel. B, Statistical analysis demonstrated that OPN-positive cells in bFGF-carrying collagen were significantly more than those in pure collagen, but they were significantly less than those in bFGF/siRNA-carrying collagen ($n = 4$, *$P < 0.05$, **$P < 0.01$).

the expression of osteogenic genes significantly increased with culture time in bFGF-containing and bFGF/siRNA-containing hydrogels(Figure 4). At day 7, the expression of ALP, OPN, BSP and OCN in MSCs from both bFGF-containing and bFGF/siRNA-containing hydrogels was significantly higher than those from control (pure hydrogel, $P < 0.05$ or 0.01, $n = 4$). In addition, ALP and BSP were significantly higher in MSCs from bFGF/siRNA-containing hydrogels than that from bFGF/ -containing hydrogels ($P < 0.05$, $n = 4$). After 14 days differentiation, these osteogenic gene levels further increased in both bFGF-containing and bFGF/siRNA-containing hydrogels compared with control ($P < 0.01$, $n = 4$). The highest expression of ALP, OPN, BSP and OCN was observed in MSCs from bFGF/siRNA-containing hydrogels, all of which were significantly higher than that from bFGF-containing hydrogels. These results indicated that bFGF/siRNA-containing hydrogels provided a superior environment for osteogenic differentiation of MSCs.

3.7 Western blotting

In addition to RT-PCR, the expression of osteogenic markers in MSCs was further analyzed from protein levels by western blotting, after 14 days differentiation in hydrogels. As shown in Figure 5, western blotting analysis has obtained consistent results with RT-PCR. Compared with control, expression of osteogenic markers, including OPN, BSP and OCN, was significantly higher in MSCs from bFGF-containing hydrogels ($P < 0.05$ or 0.01, $n = 4$). While in MSCs harvested from bFGF/siRNA-containing hydrogels, the levels of osteogenic markers were further increased and significantly higher than those from bFGF -containing hydrogels too ($P < 0.01$).

3.8 ALP activity and calcium content

After 21 differentiation, biochemical assays of ALP activity and calcium content were performed to further

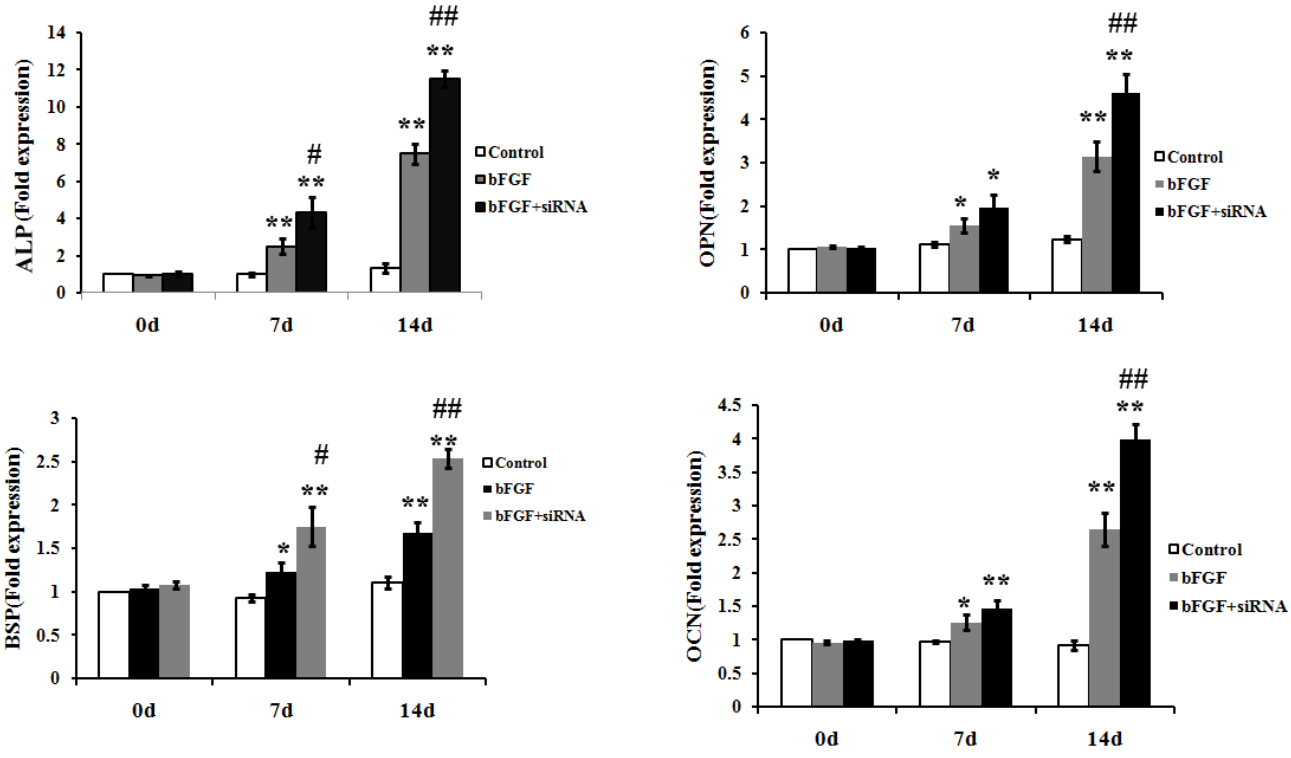

Figure 4. Osteogenic gene expression during MSC differentaition. Higher levels of ALP, OPN, BSp and OCN were detected by RT-PCR in MSCs incorporated in bFGF/siRNA-carrying collagen hydrogel compared with those in bFGF-carrying and pure collagen hydrogels (n = 4, *P < 0.05 compared with control; **P < 0.01 compared with control; #P < 0.05 compared with bFGF-carrying collagen hydrogel; ##P < 0.05 compared with bFGF-carrying collagen hydrogel).

verify the osteogenic diferentiation of MSCs. As shown in Figure 6, both ALP activity and calcium content in MSCs from bFGF/siRNA-containing hydrogels were significantly higher than those in MSCs from pure and bFGF-containing hydrogels ($P < 0.01$, $n = 4$). In comparison, ALP activity and calcium content in MSCs from bFGF-containing hydrogels were also significantly higher than those from pure hydrogel ($P < 0.01$, $n = 4$). These results provided additional evidence from protein level that bFGF/siRNA-containing hydrogels provided a superior environment for osteogenic differentiation of MSCs.

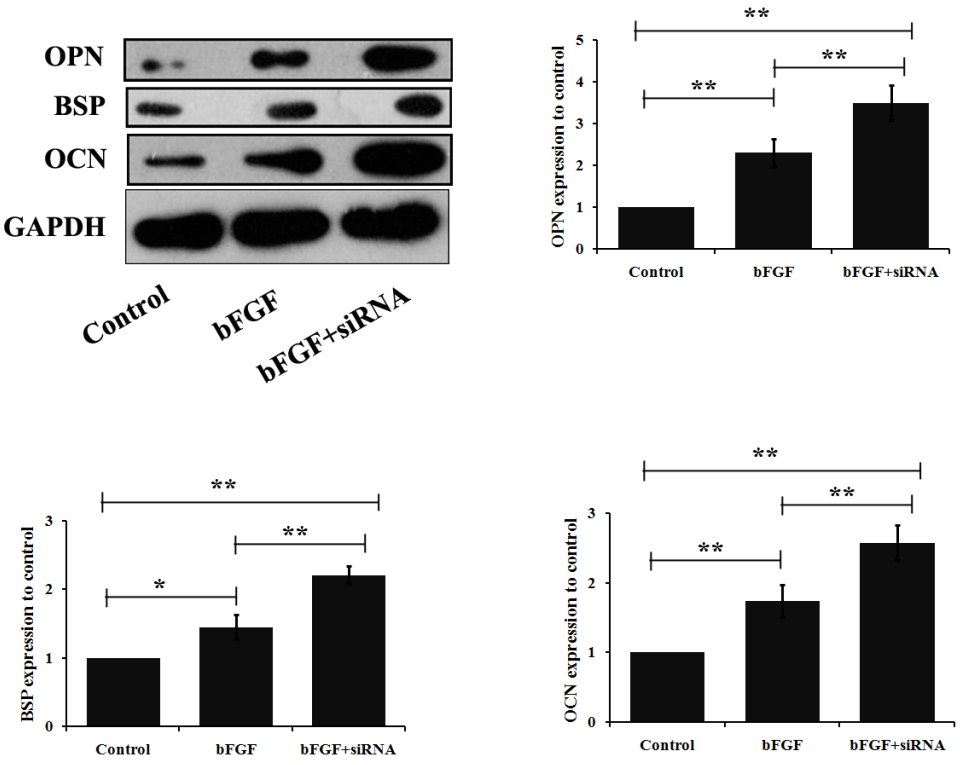

Figure 5. Western blotting of osteogenic markers. At day14, western blotting demonstrated consistent results with that of RT-PCR from protein level. Osteogenic markers, including OPN, BSP and OCN, were significantly higher in MSCs from bFGF/siRNA-carrying collagen hydrogel compared with those from bFGF-carrying and pure collagen hydrogels ($n = 4$, $^*P < 0.05$; $^{**}P < 0.01$).

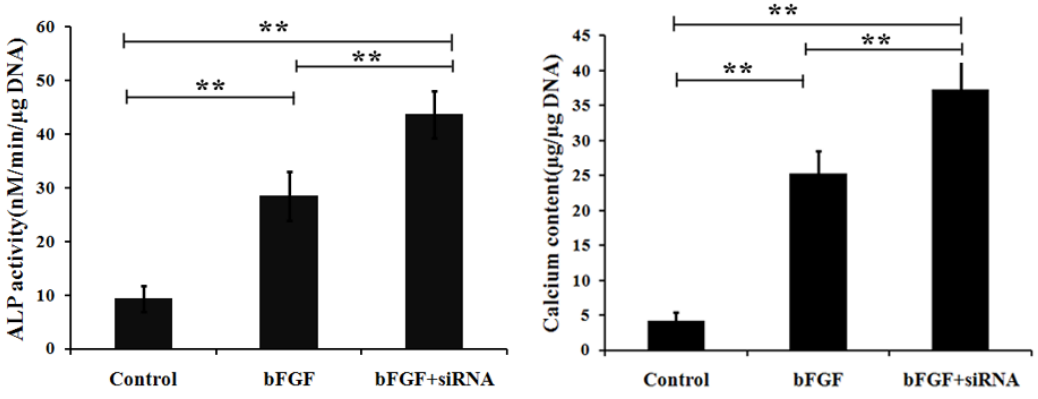

Figure 6. ALPactivity and calcium content. At day 21, biochemical assays demonstrated that both ALP activity and calcium content were higher in MSCs from bFGF/siRNA-carrying collagen hydrogel compared with those from bFGF-carrying and pure collagen hydrogels ($n = 4$, $^*P < 0.05$; $^{**}P < 0.01$).

4 Discussion

As a key component in tissue engineering, scaffolds play important roles in promoting tissue repair and thus,became a hot focus in regenerative medicine besides seeding cells [23,24]. Among various scaffolds, in situ forming hydrogels are a currently favored choice, because the seeding cells or cytokines can be easily incorporated before gel formation and furthermore, they can be efficiently retained in the target sites after delivery *in vivo* [25-27]. Among the previously reported hydrogels, collagen have gained the greatest interest [28]. In the past years, collagen as well as collagen-containing composite materials have been widely used in the field of tissue engineering, including wound dressings, skin repair, and organ tissue engineering [29,30]. As the major component of bone, collagen was confirmed to play important roles in bone repair and new bone formation. Therefore, collagen also received much attention in bone tissue engineering. To enhance the bioactivity of collagen hydrogels for bone regeneration, different strategies were explored in recent years, such as supplementing collagen with other bioactive materials or growth factors [31,32]. In the present study, we demonstrated that PPARγ inhibiting with siRNA together with bFGF could synergistically promote MSC osteogeneic differentiation. Based on the finding, we investigated the feasibility and validity of functionalizing collagen hydrogel with osteogeneic bioactivity by simultaneously incorporating bFGF and PPARγ-targeting siRNAs. We demonstrated that bFGF/siRNAs containing hydrogels kept good biocompatibility for seeding cell survival. Both bFGF and siRNAs could be efficiently retained within collagen hydrogels for more than two weeks, maintaining a longer-term effect on incorporated MSCs. From gene and protein levels, we showed that the property of the novel matrix system significantly enhanced the osteogenic differentiation of incorporated. This finding is significant in that it indicates a simple strategy to functionalize collagen scaffold with potent osteogeneic bioactivity when considering collagen hydrogel as scaffold of MSC for bone tissue engineering.

Compared with 2D culture, 3D environment is more representative of tissue architecture *in vivo* and was widely studied in recent years [33-35]. Previously, several strategies have been explored to upgrade 3D scaffolds; a typical one was introducing appropriate cytokines. As a potent osteogenic cytokine, bFGF has been confirmed in 3D culture matrix for its beneficial effects on proliferation and osteogenic differentiation of MSCs [36,37]. In the study, we observed consistent results with the previous report, that a suitable concentration of bFGF stimulated the proliferation and osteogenic differentiation of MSCs, while excess of bFGF was inhibitory [14]. The optimal bFGF was determined as 10 ng in the present study, similar to the previous report [14]. PPARγ is known as a potential target for regulating the osteogenic differentiation of progenitor cells, and suppression of PPARγ by RNA interference was demonstrated to promote osteogenic differentiation of human MSCs [2]. However, siRNA transfection with conventional method was usually transitory (within one week). Furthermore, it is hard to effectively achieve the *in vivo* siRNA delivery in clinical practice of tissue engineering with conventional methods [38,39] due to the easy degradation and loss of free siRNA molecules. As a 3D macroscopic and biopolymer scaffold, collagen hydrogel has been successfully utilized to retain siRNA locally and release it in a sustained manner to prolong the effect directly at the site of interest [19]. In the present study, we firstly reported the application of siRNA-carrying collagen hydrogel as a seeding cell matrix for tissue engineering. By carrying PPARγ-targeting siRNAs together with bFGF, we demonstrated the novel 3D scaffold was excellent in guiding osteogenesis of MSCs, promising in bone tissue engineering.

In the study, we provided sufficient evidence *in vitro* for the superiority of the novel hydrogel system in bone tissue engineering. However, the *in vivo* effectiveness was not demonstrated. This was a limit of the study, which will be a focus in our future work. In addition to regulating stem cell differentiation, the PPARγ-related signalling cascades were also critical therapeutic targets in osteoporosis and age-related osteopenia. Based on the capacities of retention and controlled release of PPARγ-targeting siRNA, the collagen hydrogels may deliver the therapeutic siRNA molecules more efficiently to target sites, and further achieve better gene therapy for osteoporosis or age-related osteopenia. Therefore, the 3D siRNA-carrying hydrogels described in the study would be useful in gene therapy for bone disorder too, which deserves an in-depth investigation.

In summary, the study reported a novel 3D hydrogel scaffold for bone tissue engineering by incorporating bFGF and PPARγ-targeting siRNAs. The developed bFGF/siRNAs containing hydrogels possessed a good biocompatibility for seeding cell survival. In the 3D hydrogel, both bFGF and siRNAs were efficiently retained for slow release, prolonging their effects on incorporated MSCs. More importantly, bFGF and PPARγ-targeting siRNAs played synergetic roles in promoting differentiation of human MSCs, significantly enhancing osteogenesis in 3D scaffold. Thus, the novel *in situ* forming hydrogel should be promising in future application in bone tissue

engineering, as well as in siRNA delivery for gene therapy of bone disorders.

Conflicts of interest: The authors confirm that there are no conflicts of interest.

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