

FGF2-adsorbed macroporous hydroxyapatite bone granules stimulate *in vitro* osteoblastic gene expression and differentiation

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Abstract Hydroxyapatite bone granules with a macroporous structure were produced and then adsorbed with basic fibroblast growth factor (FGF2). The *in vitro* scaffolding role of the granules in cell population and osteogenic differentiation was investigated. The FGF2-adsorbed porous granules allowed the MC3T3-E1 cells to adhere well and then proliferate actively. While the cell growth level on the FGF2-treated granules was observed to be similar to that on the untreated granules, the expression of genes associated with bone, including collagen type I, alkaline phosphatase, and osteocalcin was significantly up-regulated by the FGF2 treatment, particularly at the early stage. Moreover, the production of alkaline phosphatase with prolonged culturing was greatly enhanced on the FGF2-adsorbed granules. Taken together, the FGF2 treatment of the hydroxyapatite granules was effective in the osteogenic development and the FGF2-adsorbed bone granules may be useful in bone regeneration area.

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1 Introduction

As an alternative to autografts, the introduction of artificial grafting materials has allowed the successful reconstruction of bony defects [1]. The use of bone filling and augmentation materials within the alveolar ridge and periodontal pocket has recently become a popular treatment in dental implantation [1, 2]. The bone grafts developed thus far include a broad range of materials with a synthetic and natural origin [3–5]. Above all, bioactive inorganics, such as calcium phosphate ceramics (hydroxyapatite and tricalcium phosphate) and glasses with bioactive compositions have found widespread use [3–5]. The performance of bone grafts can be improved greatly through the use of bioactive molecules, such as growth factors and drugs [2]. The combined bioactive molecules have been shown to improve the *in vitro* osteogenic cellular behavior or/and *in vivo* bone forming ability [6–8]. For example, bone morphogenetic protein (BMP) and transforming growth factor (TGF- β 1) have been reported to regulate osteogenesis and bone regeneration when used in together with hydroxyapatite granules and bioactive particles [7, 8].

Basic fibroblast growth factor (bFGF or FGF2) has recently gained considerable interest in tissue regeneration on account of its specific role in angiogenesis [9, 10]. Stimulating the proliferative potential of fibroblasts and angiogenesis will speed up the closing of defective spaces in the wound healing process [9, 10]. The formation of neo-blood vessels is of particular importance in tissue regeneration of defect sites, and FGF2 plays a key role in the osteogenesis and bone formation process [11, 12]. Recent studies have shown that the use of FGF2 in bone defects improves the bone formation rate in association with early angiogenesis [12].

Recently, the authors synthesized recombinant FGF2 containing amino acid sequences almost identical to the native one [13], which is known to play a vital role in angiogenesis [14]. When an optimal concentration within a culturing medium was chosen, significant improvement in the proliferation and differentiation of osteoblastic cells was noticed to a level comparable to a native FGF2 treatment. The aim of this study is to utilize the recombinant FGF2 in conjunction with hydroxyapatite granules, which are particularly designed to retain interconnected macro-channels within the structure. The *in vitro* cell growth and osteogenic behavior upon the FGF2-treated HA granules is examined, and the results may be useful for future applications of the FGF2-HA porous granules in the bone reconstruction area.

2 Materials and methods

2.1 Macroporous hydroxyapatite (HA) granules

Macroporous HA granules were produced via a freeze-casting method using camphene as a porogen, as described previously with a slight modification [15]. Briefly, commercially available hydroxyapatite (HA) powder (Alfa aesar) calcined at 900°C was mixed with camphene (C₁₀H₁₆, Aldrich) at an appropriate ratio (HA/Camphene = 20 wt%), and the oligomeric polyester Hypermer KD4 (UniQema) at 5 wt% was added as a dispersant. The HA/camphene slurry was ball-milled at 60°C for 24 h and then cast into a mold at 35°C to allow solidification, and stored at -20°C for additional 24 h. The frozen blocks were left under a laminar flow to induce camphene sublimation. The camphene sublimation could be monitored by the weight loss, and the complete sublimation was determined by the end point of weight loss of the green body (within 24 h under laminar flow). The blocks were cut into small pieces (~a few millimeters) and then heat-treated to 1,250°C for 3 h. Samples, 1,000–2,000 μm in size, were chosen by sieving selectively for further tests. For the protein adsorption and cell tests, granules were sterilized with 70% ethanol and fully dried under a sterilized condition. The microstructure of the granules was observed with scanning electron microscopy (SEM, Hitachi).

2.2 Recombinant fibroblast growth factor 2 (FGF2)

FGF2 was designed, as described elsewhere [13]. Briefly, the cDNAs of the basic fibroblast growth factor were amplified from an adult human cDNA library. According to the GenBank™ sequence (GenBank™ accession number NM002006), a pair of primers, 5'-CGAGATCTCAGCCG GGAGCATCAC-3' and 5'-TGCAGATCTCGCTCTTAGC

Table 1 Primer sequences of the bone-associated genes used in real time PCR

Gene	Primer sequence
Collagen type I	Forward: 5'-GCATGGCCAAGAAGACATCC-3' Reverse: 5'-CCTCGGGTTTCCACGTCTC-3'
Alkaline phosphatase	Forward: 5'-GCATGGCCAAGAAGACATCC-3' Reverse: 5'-CCTCGGGTTTCCACGTCTC-3'
Osteocalcin	Forward: 5'-AAGCAGGAGGGCAATAAGGT-3' Reverse: 5'-TTTGTAGGCGGTCTTCAAGC-3'
Osteopontin	Forward: 5'-GACCACATGGACGACGATG-3' Reverse: 5'-TGGAACCTGCTTGACTATCGA-3'
GAPDH	Forward: 5'-CCCTGTTGCTGTAGCCGTA-3' Reverse: 5'-CCGGTGCTGAGTATGTCG-3'

AGACATTG-3', was designed and used. The PCR products were cloned into pBAD/His A (Invitrogen, Carlsbad, CA) in-frame using the NH₂-terminal 6X His tag. The recombinant FGF2 protein containing the poly-His tag was expressed and purified using a Ni²⁺ affinity column under denaturing conditions according to the manufacturer's protocol (Invitrogen) (Table 1).

2.3 FGF2 adsorption to HA granules and his-tag probe assay

HA granules (~100 mg) were placed in each well of a 24-well plate and adsorbed with a 0.25 μM FGF2 solution for 5 h at room temperature. The FGF2-HA granules were exposed to a horseradish peroxidase (HRP)-conjugated His antibody (Santa Cruz Biotechnology) for 1 h at 37°C. A colorimetric substrate for the HRP was then added (Pierce). After dissolving, the sample was read at an absorbance 450 nm using a spectrophotometer. Moreover, the fluorescent image of the FGF2 adsorption to the granules was detected under confocal laser scanning microscopy (CLSM; LSM510, Carl Zeiss).

In order to assess the stability and release of FGF2 from the HA granules, the FGF2-HA granules were incubated in an acellular culture medium for up to 3 days, and the his-tag probe assay was performed to measure the remaining FGF2. The fluorescence observation of the FITC-conjugated FGF2 was carried out using CLSM.

2.4 Cell culturing and growth

The MC3T3-E1 osteoblastic cells were maintained in α-MEM (Invitrogen) containing 10% heat-inactivated fetal calf serum (Invitrogen), 100 units/ml penicillin G sodium, 100 μg ml⁻¹ streptomycin sulfate, and 0.25 μg ml⁻¹ amphotericin B (Invitrogen) in a 5% CO₂ atmosphere at 37°C. When the cells in a culture flask reached confluence,

they were detached with 0.25% trypsin/1 mM ethylenediamine tetraacetic acid (EDTA), counted and suspended in a culture medium at a density of 5×10^4 /ml. Before cell seeding, the HA granules were sterilized with 70% ethanol, dried and placed onto each well either of 24- or 96-well plate. The amount of HA granules was determined to almost cover the culture well in monolayer, which corresponds to 15 mg for 96-well and 100 mg for 24-well. One set of HA granules contained in the well was treated with FGF2, as described above. Granules for the cell test were washed with acellular culture medium twice and an aliquot (150 μ l for 96-well and 1 ml for 24-well) of the cell suspension was seeded onto each granule sample, and incubated for different periods. The culturing medium was refreshed twice a week.

The cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) method. This assay is based on a colorimetric measurement of the water-soluble formazan product created by reduction of the MTS reagent through the mitochondrial activity in viable cells. At each culturing period, a 20 μ l aliquot of the CellTiter 96 Aqueous One Solution Reagent (Promega, Madison) was added to each sample, according to the manufacturer's instructions. The cell viability was determined from the absorbance at 490 nm using an Elisa Plate Reader.

2.5 Cell morphology observation

The cell morphology was observed by scanning electron microscopy (SEM, Hitachi). After culturing, the cells were fixed with 2.5% glutaraldehyde, dehydrated in a graded series of ethanol (50, 70, 90, 95 and 100%), and treated with a hexamethyldisilazane (HMDS) solution. After coating the samples with Pt, the electron microscope was operated in normal mode at an accelerating voltage of 15 kV.

2.6 Alkaline phosphatase determination

To determine the activity of alkaline phosphatase (ALP), MC3T3-E1 cells were cultured on the HA and FGF2-adsorbed HA granules for 1, 2, and 3 weeks. After incubation, the cells were washed with PBS and lysed in 1.5 M Tris-HCl, pH 10.2 containing 1 mM ZnCl₂, 1 mM MgCl₂ and 1% Triton X-100 at 4°C for 10 min. After clarifying the cell lysates by centrifugation, the ALP activity was determined using an ALP assay kit (Procedure No. ALP-10, Sigma) for 60 min at 37°C. A *p*-nitrophenol, which is yellow in color, is produced in the presence of ALP. Its absorbance was determined spectrophotometrically at 405 nm using a microplate reader (Molecular Devices). The ALP activity was normalized to the total protein

content of each sample using a Protein Assay Kit (Invitrogen).

2.7 Gene expression by real time PCR

The MC3T3-E1 cells were cultured onto the HA and FGF2-adsorbed HA granules for up to 14 days. The RNA was extracted using an Easy-spin RNA Extraction kit (iNtRON, Korea) according to manufacturer's instructions. After isolation, the RNA was converted to cDNA and amplified. Accumulation of the PCR products was monitored and quantified using SYBR GreenER qPCR SuperMix reagents (Invitrogen) and a comparative CT method, in which the accumulated PCR products for each gene examined (collagen type I, alkaline phosphatase, osteocalcin and osteopontin). Housekeeping gene GAPDH was used as reference. The up- and down-regulation of gene expression was monitored. The primer sequences of the bone-associated genes used in real time PCR are presented in Table 1.

2.8 Statistics

Data are expressed as the means \pm one standard deviations on five different sets of experimental samples for each group. Statistical analysis was carried out by Student's *t* test. Statistical significant was considered at $P < 0.05$.

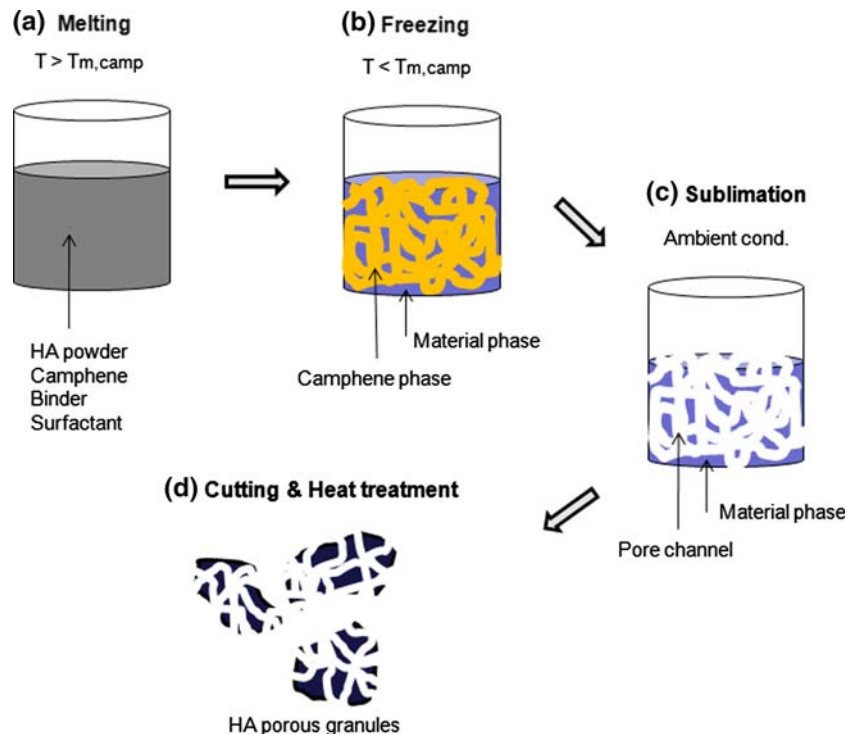
3 Results

3.1 Macroporous HA granules and FGF2 adsorption

Figure 1 shows the processing route to produce macroporous HA granules. As depicted, the camphene used as a porogen was mixed with the HA slurry containing the binder and surfactant at temperatures higher than the melting point of camphene ($T_m \approx 40^\circ\text{C}$) (Fig. 1a). When the mixture was cooled to just below the freezing point of camphene (down to $\approx 35^\circ\text{C}$), it solidified to form a continuous biphasic mixture of camphene and HA (Fig. 1b) [15]. Further sublimation of camphene under ambient conditions resulted in the generation of continuous pore channels and remnant HA (Fig. 1c). Final heat-treatment was needed to remove the organic phase and sinter the HA powders to provide structural and mechanical integrity (Fig. 1d). Granules, ranging in size from 1 to 2 mm, were selected for further testing.

Figure 2 shows the morphology of the HA granules obtained by a freeze-casting method. Interconnected macropores (over 100 μm in size) were well developed within the granules (Fig. 2a). The surface microstructure showed a fully densified polycrystalline structure consisting of grains less than a few micrometers in size (Fig. 2b). The

Fig. 1 Schematic diagram of the processing route to produce macroporous HA bone granules: **a** melt mixture of camphene porogen in concert with the HA powder, binder and surfactant was **b** cooled to below the freezing point of camphene (<40°C) to solidify the camphene and to form a continuous biphasic mixture of camphene and the material. **c** Sublimation of camphene under ambient conditions generated pore channel and the remnant material phase. **d** The material phase was cut to appropriate dimensions and heat-treated to remove the organic binder and to consolidate the HA macroporous granules. Illustration was referred to [14]



macropores were fully interconnected and the porosity was measured to be approximately 88%.

The HA granules were soaked in a FGF2 solution, and the adsorbed FGF2 was observed by a confocal microscopy, as shown in Fig. 3a. The FITC-conjugated FGF2, which was revealed in green, showed an even distribution over the HA granule surface. The adsorbed FGF2 was further soaked in an acellular culture medium for up to 3 days to determine the adsorption stability. Approximately 80% of the initially adsorbed FGF2 remained without being released over 3 days (Fig. 3b).

3.2 Osteoblastic responses to FGF2-HA granules

The osteoblastic responses to the FGF2-HA granules were examined by determining the level of cell growth, gene expression and differentiation. Figure 4 shows the cell growth morphology on the HA granules with or without FGF2 adsorption. For both samples, the cells spread and grew actively over the surface of granules (Fig. 4a). Compared to that on HA, the cell layer on FGF2-HA was shown to be less homogeneous, containing more aggregates and fibrous structure. The cell viability was assessed using an MTS method (Fig. 4b). The results showed comparable initial adhesion (day 0) and an on-going increase with culturing time for up to 14 days. The level of cell growth appeared to be slightly higher on FGF2-HA than on HA, but the difference was not significant ($P > 0.05$ by student *t* test).

The gene expression of the cells cultured for different culturing times was measured by real time PCR, as shown in Fig. 5. A series of bone-associated genes, including collagen type I (Col I), alkaline phosphatase (ALP), osteocalcin (OCN), and osteopontin (OPN), were stimulated on the FGF2-HA with respect to the native HA granules. In particular, up-regulations were more noticeable for the Col I, ALP and OCN genes at the initial culturing period (for 1 and 3 days, $*P < 0.05$). At prolonged culturing time, the difference was not so significant.

The ALP activity produced by the cells after culturing for up to 3 weeks was assessed, as shown in Fig. 6. After 2 and 3 weeks, significantly higher ALP level was observed on the FGF2-HA granules ($*P < 0.05$).

4 Discussion

The reconstruction of alveolar bone defects is of particular importance in dentistry for successful implantation surgery. The limited volume of bone in both width and length caused by trauma or disease should be increased and augmented to provide sufficient space for implantation [1, 16]. The use of bone grafts in concert with biologically active molecules, such as bone morphogenetic proteins and growth factors, has become a possible alternative to improving the quantity and quality of augmented bones [2]. Moreover, tissue engineering through artificial materials with progenitor/stem cells is considered as a promising

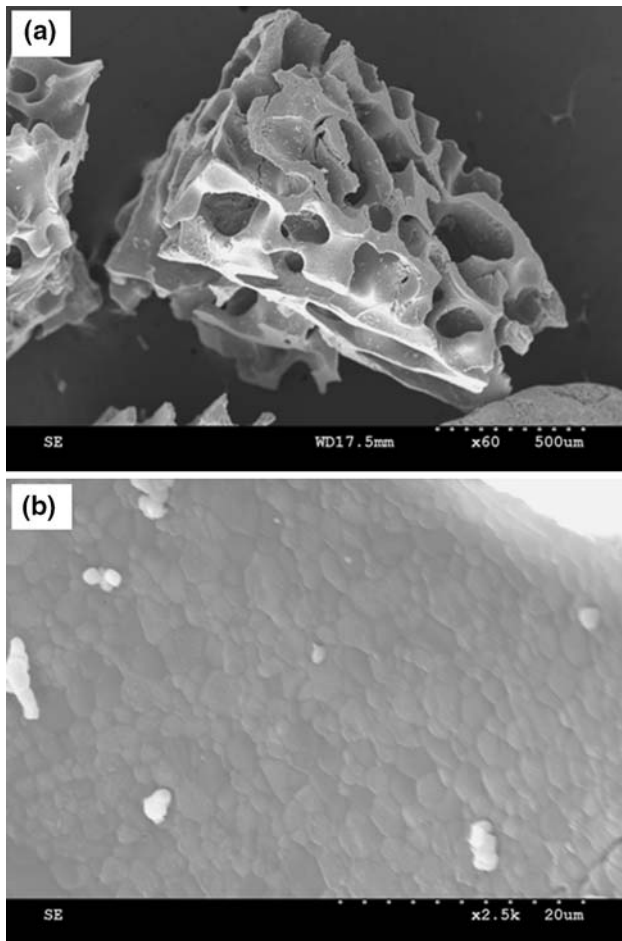


Fig. 2 SEM morphology of the HA granules used for the adsorption of FGF2: **a** low magnification image showing the macroporous HA granules and **b** high magnification image of the surface microstructure revealing grains, with a few micrometers in size, which are typical of sintered polycrystalline HA. Macropores within the HA granules were generated by the freeze-casting method (as illustrated in Fig. 1)

direction for the reconstruction of alveolar bone [17]. As a first step toward tissue engineering of alveolar bone in dentistry, the authors engineered bioceramic bone granules in combination with bioactive molecule fibroblast growth factor (FGF2) to improve the osteogenic ability of porous granular scaffolds.

In particular, interconnected macropores were introduced within the bone granules by a novel method in order to provide channels for the blood and nutrient supply as well as a large space and area for biological reactions and a cellular population, which improves the scaffolding potential [18, 19]. As shown in Fig. 1, well-developed channeled macropores were provided by the freeze-casting method using a camphene as the porogen. As the solidified camphene is converted directly to the pore part of the bone granules, its content and freezing characteristics are vital for determining the porosity and pore geometry. In particular, in order to generate large-sized pores (about over

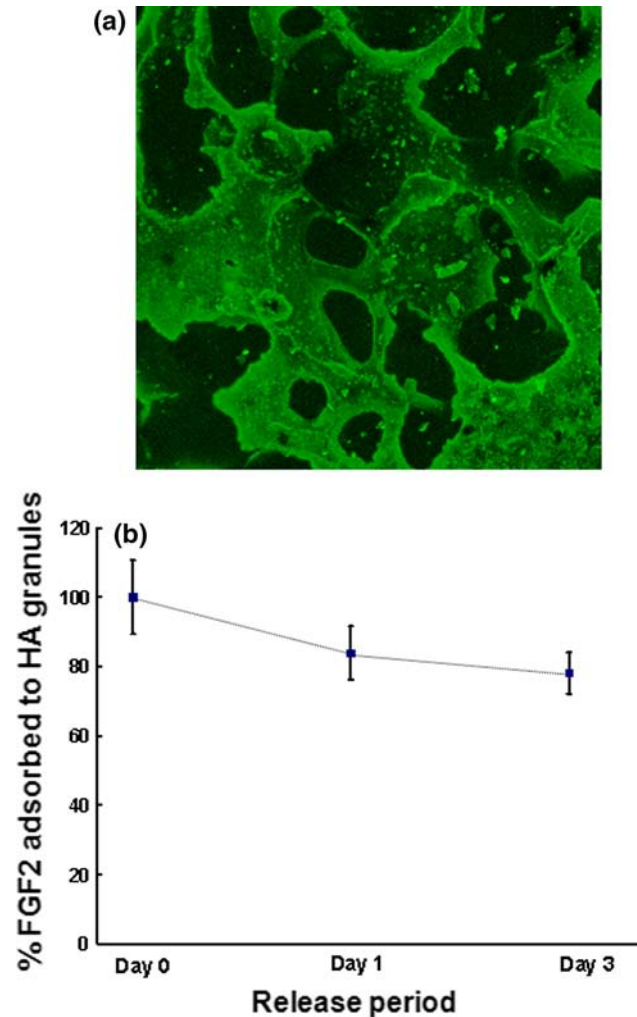


Fig. 3 **a** Confocal laser scanning microscopy image of the FITC-conjugated FGF2 stained in green, showing that the adsorbed FGF2 almost fully covered the surface of the HA granules. **b** Release profile of the FGF2 from the HA granules during incubation in an acellular culture medium for up to 3 days, as assessed by a his-tag probe. Approximately 80% of the initially adsorbed FGF2 remained at least for 3 days of incubation

100 µm), the freezing temperature should be high, i.e., just below the freezing point of camphene (herein 35°C), by which the nucleation of camphene is impeded while the growth process is accelerated [15]. The macropores present in the developed bone granules are believed to provide sufficient space for cell migration and population in bone tissue engineering applications.

The surface of the bone granules was conjugated with FGF2 to provide more bioactive substrate conditions for the cells to populate on and further synthesize the appropriate extracellular matrix for bone tissue formation. FGF2, as a signaling molecule, plays important roles in controlling various biological functions, including cell proliferation, migration, and differentiation [9]. Mutations in the FGF-receptors are closely related to many human genetic

Fig. 4 a MC3T3-E1 cell morphology grown on the HA granules with and without FGF2 adsorption after 3 days of culturing. The cells appeared to show active adhesion and spreading on the granules.
b MTS assay on the cell viability, showing the adhesion for 24 h (day 0) and growth for 7 and 14 days. Data showed a slightly higher level on FGF2-HA with respect to pure HA granules but there was no significant difference between the two materials ($n = 5$, $P > 0.05$ Student's t test)

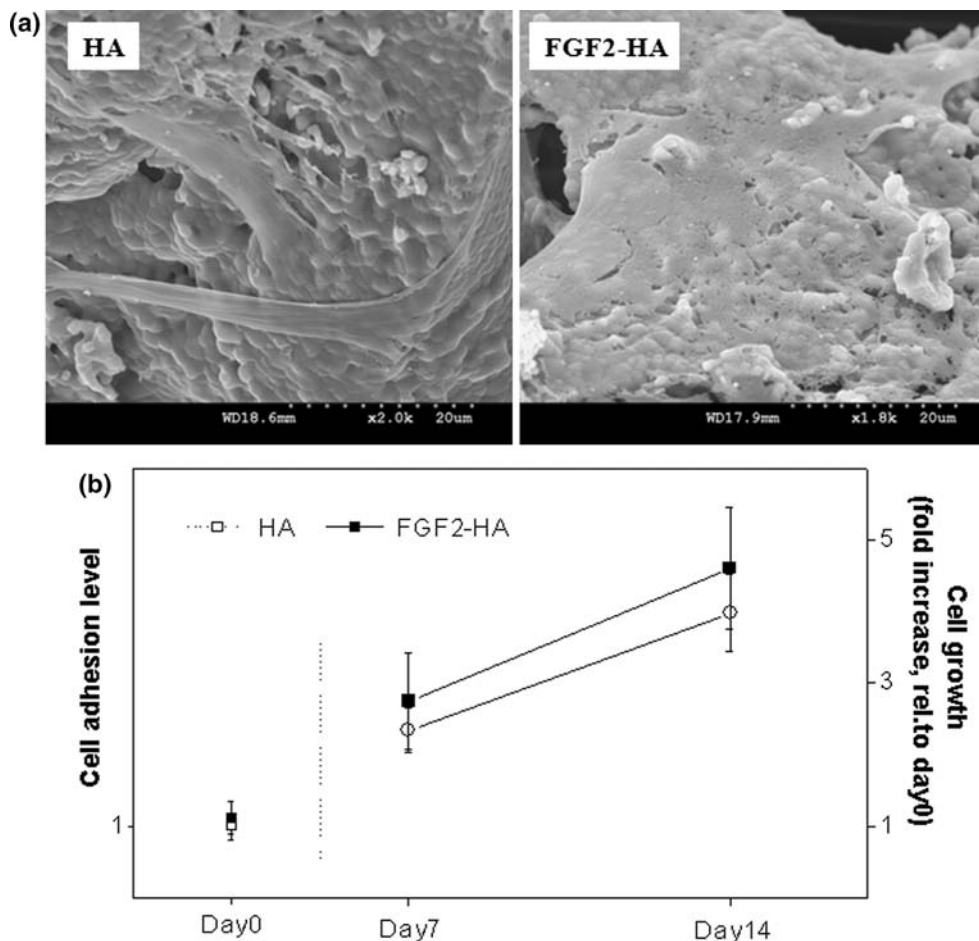
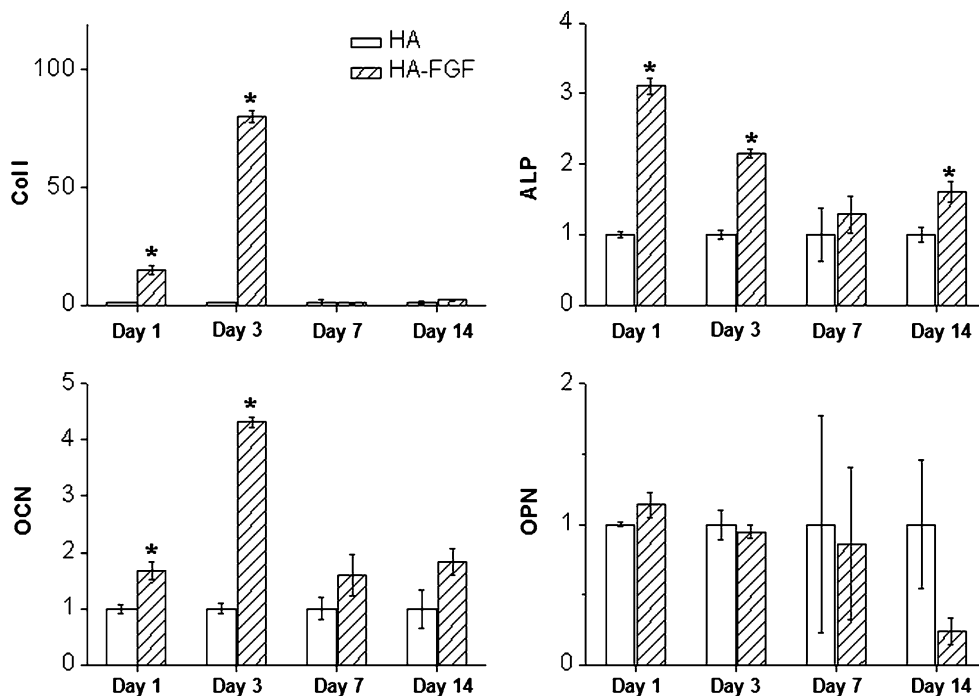


Fig. 5 Real-time PCR gene expression on the HA granules with and without the adsorption of FGF2 at days 1, 3, 7 and 14. The bone-associated genes, including collagen type I (Col I), alkaline phosphatase (ALP), osteocalcin (OCN) and osteopontin (OPN), were assessed, and data were presented with respect to those without FGF2. The expression was significantly higher on the FGF2-HA granules with respect to the HA granules ($* P < 0.05$ by Student's t test)



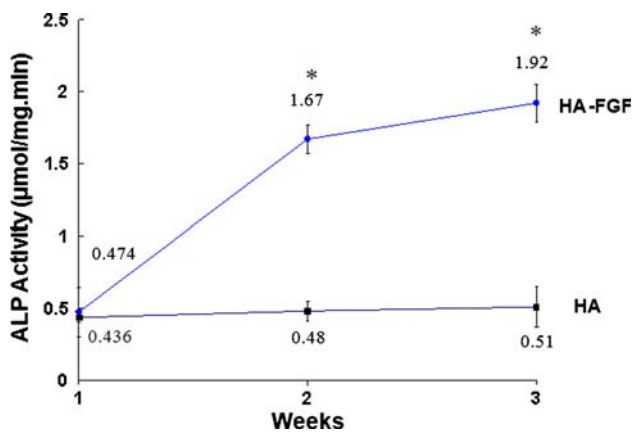


Fig. 6 ALP activity of the cells grown on the HA granules with and without the adsorption of FGF2, after 7, 14 and 21 days of culturing. A significantly higher level was observed at 2 and 3 weeks (* $P < 0.01$ by Student's t test)

diseases involving the severe impairment of bone formation, including dwarfism, providing evidence that FGF signaling is important in the bone metabolism [20]. In vitro cellular studies have shown that FGF2 stimulates the proliferative potential and angiogenesis of cells [21]. Because of the special importance of neo-vascularization in bone regeneration, the biological role played by FGF2 is considered to be favorable for osteogenesis and bone formation [11]. Some studies utilizing FGF2 in concert with biomaterial scaffolds demonstrated effective angiogenesis and rapid bone defect closure [12, 22, 23].

From a pilot study, we observed that the recombinant form of FGF2 used in this study significantly enhanced the osteoblastic proliferation when treated directly within a medium on the culture dish at a dose of 20 ng/day; the MTS cell viability level was 0.853 ± 0.131 (treated) and 0.402 ± 0.024 (untreated) at day 3 and 1.662 ± 0.129 (treated) and 0.772 ± 0.062 (untreated) at day 6. Based on this, in this study, FGF2 was utilized onto the surface of the macroporous HA bone granule, which is more relevant to the clinical applications. As revealed by his-tag probing under fluorescence image, FGF2 was shown to adsorb easily onto the macroporous HA granules. Moreover, the adsorbed protein maintained its initial quantity, up to ~80% for at least 3 days in a culturing medium (Fig. 3). This suggests that FGF2 adsorption to the HA surface is quite selective and irreversible. Therefore, FGF2 is expected to play roles in eliciting effective biological activity to the cells including cell mitosis and osteogenic differentiation.

Interestingly, the adsorption of FGF2 onto the HA granular surface did not have any significant impact on cell growth, as revealed by the cell growth morphology and viability (Fig. 4). Given that many studies insist that FGF2 stimulates cell mitosis and proliferative potential, its role in

the macroporous HA granules was not so obvious, which is possibly due to the surface condition of the HA granules used herein, i.e., being quite favorable for the cell migration and mitosis. However, the expression of genes associated with bone at the initial stages was significantly up-regulated by the FGF2 adsorption. More than 2–5 fold increases in the collagen I, alkaline phosphatase and osteocalcin were noticed on the FGF2-adsorbed HA granules, particularly at the early stage of culturing (~day 3). Furthermore, significant alkaline phosphatase (ALP) synthesis was observed on the granules by the FGF2 conjugation, suggesting the cells grown on the FGF2-tethered surface were better triggered to undergo osteogenic development at later stage.

With regard to ALP, the initial gene expression and further synthesis were both up-regulated. Based on the stability of FGF2, which remained adhered to the HA particles for at least up to 3 days (Fig. 3), the altered gene expression might be affected by the surface-adsorbed FGF2 through the action of the FGF-receptor, which is followed by the intracellular signaling process and gene expression [13]. In this manner, the ALP amplification at the later stage (2–3 weeks) may in part be the result of an intracellular signaling process. Therefore, the initial significantly altered expression of bone-associated genes highlights the possible role of FGF2 in the further osteogenic differentiation and synthesis of bone extracellular matrix molecules, which however remains as future study. Although limitedly performed within an in vitro regime, this study showed that the newly developed bone granules containing macropores can be combined with FGF2 biomolecules and then be used to promote biological activity in the osteogenic differentiation. However, a further in vivo study will be needed to clarify the potential of the FGF2-conjugated bone granules in supporting the regeneration of bone.

5 Conclusions

Macroporous hydroxyapatite bone granules produced by a freeze-casting method were adsorbed with basic fibroblast growth factor (FGF2) to improve the scaffolding role in osteogenic differentiation. MC3T3-E1 cells favored the FGF2-treated granule surface, proliferating actively with a similar growth level to that of the untreated granule surface. The initial gene expression, coding alkaline phosphatase, collagen I and osteocalcin, was up-regulated significantly by the FGF2 adsorption. Furthermore, alkaline phosphatase activity was better stimulated on the FGF2-HA granules than on the native HA with prolonged culturing, suggesting the role of FGF2 in the osteogenic development. These results suggest that the developed

macroporous HA granules in combination with FGF2 may be useful as a scaffold for bone regeneration area.

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