Int J Clin Exp Med 2017;10(2):2127-2136
www.ijcem.com /ISSN:1940-5901/IJCEM0028257

Original Article

10% HA/SPEEK/PEEK composite promotes proliferation and differentiation of osteoblast cells through the MAPK and PI3K/AKT signaling pathways

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Received March 15, 2016; Accepted September 19, 2016; Epub February 15, 2017; Published February 28, 2017

Abstract: Hydroxyapatite (HA) is a material that is similar to the inorganic composition of bone. Polyetheretherketone (PEEK) has the suitable physical and chemical properties, but with bioinertia. In the present study, the bioactivity of 10% HA/SPEEK/PEEK composite on the proliferation and differentiation of osteoblast cells as well as the underlying mechanism was explored. 10% HA/SPEEK/PEEK composite was found to promote the proliferation of osteoblast cells and regulate their differentiation. Further study showed that the activation of ERK, JNK, P38 signaling pathways and the PI3K/AKT signaling pathway was involved in the effects of 10% HA/SPEEK/PEEK composite. Our study suggests that 10% HA/SPEEK/PEEK composite may be a promising material to be used as a bone-implant material.

Keywords: 10% HA/SPEEK/PEEK composite, proliferation, differentiation, osteoblast cells, ERK, JNK, P38, PI3K/AKT

Introduction

Hydroxyapatite (HA), a synthetic calcium phosphate biomaterial whose main composition is $Ca_{10}(PO_4)_6(OH)_2$, is similar to the inorganic composition of bone. HA has excellent biocompatibility and bioactivity and has widely application [1]. However, due to the hard texture, great brittleness and poor toughness, the application of HA is limited. Nano-hydroxyapatite (nano HA) shows high mechanical strength and excellent biocompatibility. Nano HA was discovered to promote the proliferation and invasion of osteoblast effectively [2, 3]. Nano HA was also reported to up-regulate the expression of fibroblast growth factor-2 (FGF-2), regulate the expression of endothelial constitutive nitric oxide synthase (ecNOS) and cycloxygenase-2 (COX-2), and enhance the response to vascular endothelial growth factor (VEGF), thus affecting vascular function [4, 5]. Due to the inherently osteoinductive potential, nano HA was usually chosen as the coating material. Coating with nano HA was reported to enhance the osteoinductive potential of materials, making them more suitable for applications in bone tissue engineering [6].

Polyetheretherketone (PEEK) is a semi-crystalline thermoplastic material. PEEK has stable physical and chemical properties [7]. PEEK shows abrasion resistance and high temperature resistance [8], and is used in situations in which robustness and chemical resistance at high temperatures are required. PEEK is bone-implant material used in orthopedic applications [7, 9]. Moreover, the engineering properties of PEEK are similar to those of cortical bone [10]. However, PEEK has no effect onosteoblasts [10, 11]. The bioinertia of PEEK limits its application.

Bakar and colleagues [12] tried to add HA into PEEK to empower PEEK the bioactivity. However, due to the big particle size of HA and the high dosage, the mechanical strength of PEEK was low down. In the present study, we added nHA to PEEK to form 10% HA/sulfona-

Table 1. Sequence of primers for quantitative real time PCR

Gene name	Forward primer (5'->3')	Reverse primer (5'->3')
ALP	ATGAAATACGAGATCCACCGAGAC	ATGCGACCACCCTCCACGAA
COLI	AGGTGTTGTGCGATGACGTGAT	TGGTTTCTTGGTCGGTGGGTGA
β-actin	CTTAGTTGCGTTACACCCTTTCTTG	CTGTCACCTTCACCGTTCCAGTTT

tion polyetheretherketone (SPEEK)/PEEK composite. The effects of 10% HA/SPEEK/PEEK on the proliferation and differentiation of osteoblast cells as well as the underlying mechanism were explored in the present study.

Materials and methods

Extracts preparation

PEEK, SPEEK/PEEK, HA, and 10% HA/SPEEK/PEEK powder were obtained from Shenzhen University (Shenzhen, China). PEEK, SPEEK/PEEK, HA, and 10% HA/SPEEK/PEEK was added into serum-free RPMI1640 medium (Gibco, Grand Island, NY, USA) respectively to form suspensions (8 mg/ml). Then the suspensions were filtrated for sterilization after immersion for 48 h. 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA) was then added and these mixtures were used for subsequent cell culture.

Cell culture

Human osteoblast cell line MG63 was cultured in RPMI1640 medium supplemented with 10% FBS and maintained in a humidified atmosphere at 37°C with 5% CO_2.

Cell proliferation assay

Cells were treated with extracts of PEEK, SPEEK/PEEK, HA, 10% HA/SPEEK/PEEK and normal RPMI 1640 medium (blank) for 48 h. Then cells were harvested by centrifugation and washed with phosphate buffered saline (PBS). Cells were fixed in ice-cold 70% ethanol for 2 h at 4°C. After washing with PBS, cells were stained with a Cell Cycle Detection Kit (Beyotime, Shanghai, China) at 37°C for 30 min in the dark. Then cells were analyzed with flow cytometer (BD, Franklin Lakes, NJ, USA). Proliferation index (PrI) was calculated by the following formula: PrI = the percentage of cells in S phase + the percentage of cells in G2/M phase.

Quantitative real time PCR (qRT-PCR)

Cells were treated with extracts from different composites or blank for 14 d, 21 d, and 28 d. Then total RNA was extracted using Total RNA Extraction Kit (Tiangen, Beijing, China) and reverse transcribed to cDNA using Super M-MLV reverse transcriptase (BioTeke, Beijing, China). Then mRNA levels of alkaine phosphatase (ALP), collagen I (COLI) were measured using SYBR Green method with primers in **Table 1**. Quantitative real time PCR was performed on an Exicycler TM 96 quantitative PCR instrument (BIONEER, Daejeon, Korea). The SYBR Green reagent was obtained from Solarbio (Beijing, China). Relative mRNA level was calculated using the $2^{-\Delta\Delta Ct}$ method [13].

Western blot

Cells were collected and protein was extracted using radio immunoprecipitation assay (RIPA) lysis buffer. After measurement of protein concentration using a BCA Protein Assay Kit (Beyotime), equal amount of protein was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Then the separated protein was transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA). After blockade with 5% skim milk or bovine serum albumin (BSA), the PVDF membranes were incubated with corresponding primary antibodies against extracellular signal-regulated kinase (ERK), phosphorylated ERK (p-ERK), c-Jun N-terminal kinase (JNK), phosphorylated JNK (p-JNK), P38, phosphorylated P38 (p-P38), phosphorylated phosphatidylinositol 3-kinase (p-PI3K) (1:500, Bioss, Beijing, China), PI3K (1:400, Boster, Wuhan, China), protein kinase B (AKT), phosphorylated AKT (p-AKT) (1:200, Santa Cruz, Dallas, TX, USA) and β-actin (1:1000; Wanleibio, Shenyang, China) at 4°C overnight. After washing with Tris buffered saline with Tween (TBST), the PVDF membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:5000; Beyotime) at 37°C for 45 min. Then the targeted protein was detected using an Enhanced Chemiluminescence (ECL) Detection System (7sea Biotech, Shanghai, China) and the optical density was analyzed using Gel-Pro-Analyzer software.

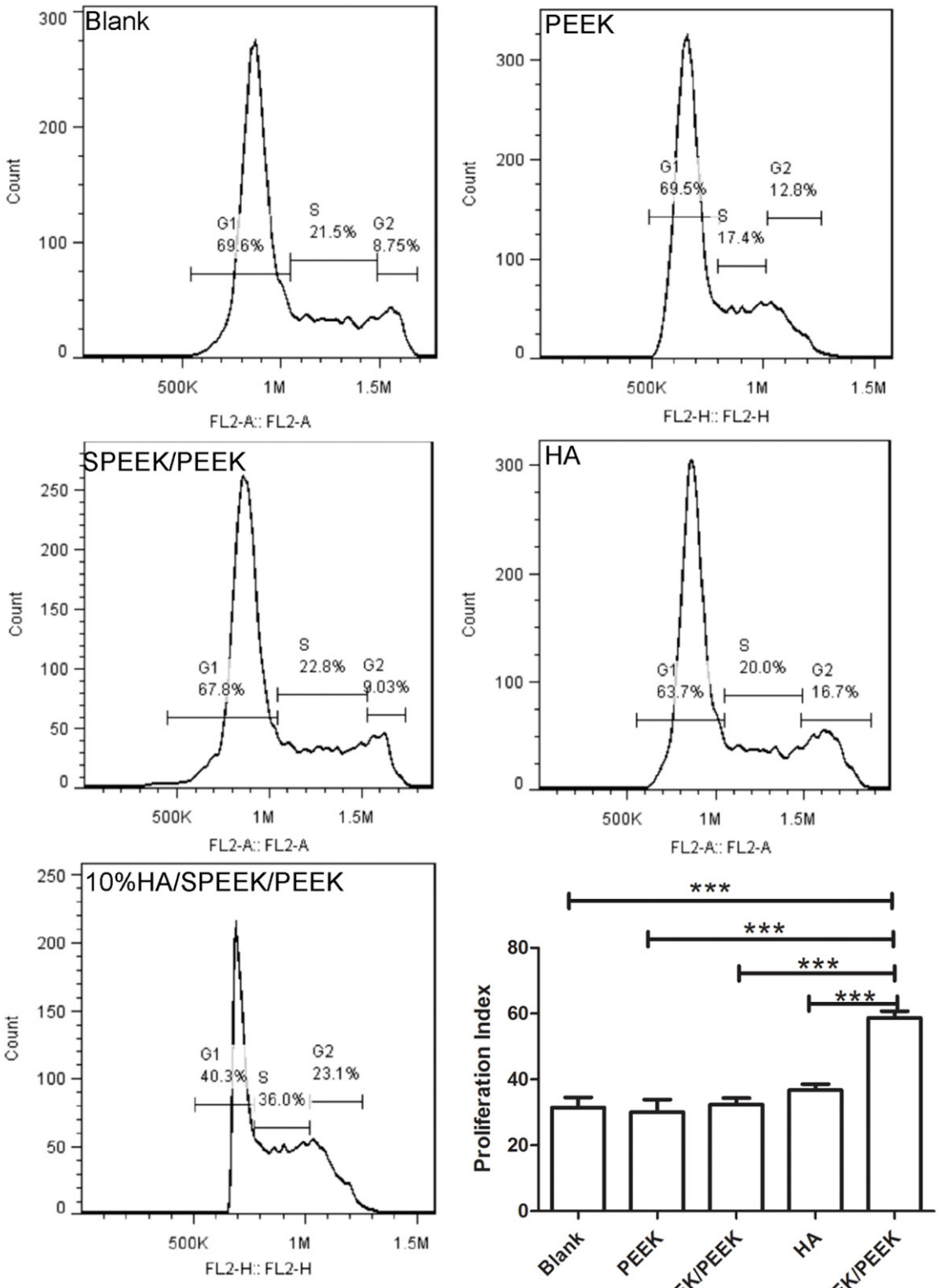

Figure 1. 10% HA/SPEEK/PEEK composite promoted the proliferation of osteoblast cells MG63. After treatment with extracts of different composites, the proliferation index was detected by flow cytometry. Each experiment was repeated three times, and the results are presented as mean ± SD. ***P<0.001.

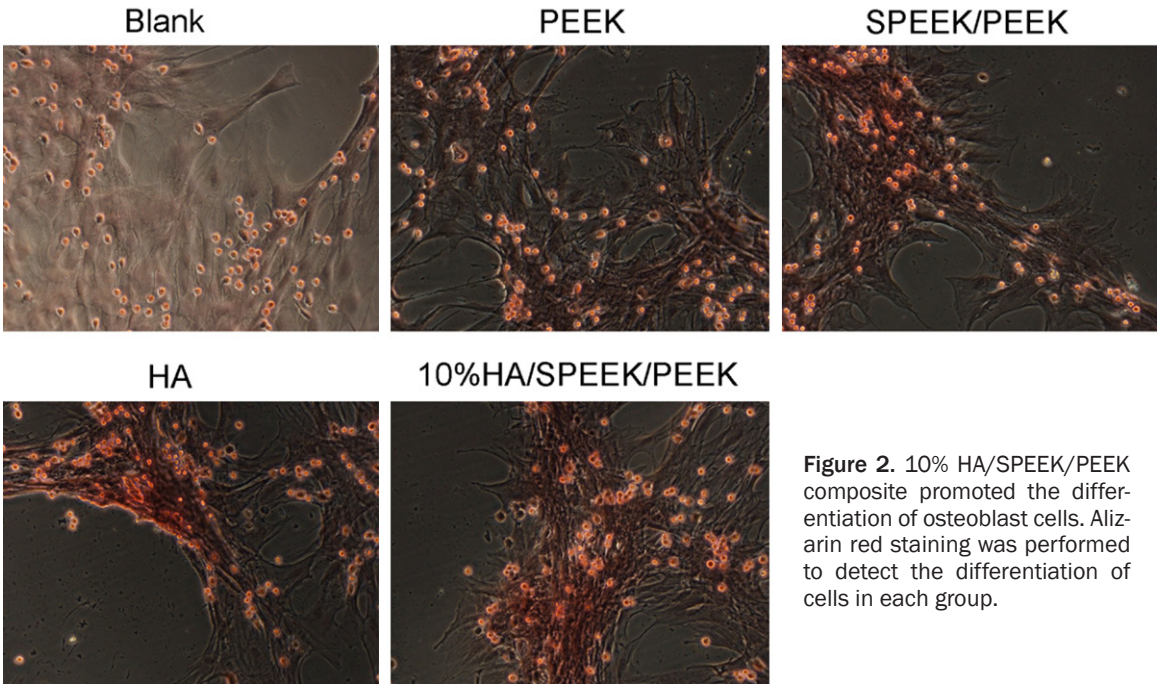

Figure 2. 10% HA/SPEEK/PEEK composite promoted the differentiation of osteoblast cells. Alizarin red staining was performed to detect the differentiation of cells in each group.

Alizarin red staining

Cells were treated with extracts of PEEK, SPEEK/PEEK, HA, 10% HA/SPEEK/PEEK and blank for 14 days. Then the cells were fixed with 70% ethanol for 1 h. Thereafter, the cells were washed with PBS twice and stained with 0.5% alizarin red at room temperature for 1 h. After washing with PBS, the cells were observed under an optical microscope with a 400× magnification.

Statistical analysis

All experiments were repeated three times. Results are presented as mean ± SD. Differences between each group were analyzed using One-way Analysis of Variance followed by Bonferroni's Multiple Comparison. $P<0.05$ was considered to be significant.

Results

10% HA/SPEEK/PEEK composite promoted the proliferation of MG63

After incubation with extracts of PEEK, SPEEK/PEEK, HA, and 10% HA/SPEEK/PEEK for 48 h, the proliferation of MG63 was detected by flow cytometry and the proliferation index was calculated. Results showed that extracts of PEEK, SPEEK/PEEK, and HA had no influence on the proliferation of MG63 cells. However, cells incubated with extract of 10% HA/SPEEK/PEEK showed a significant change, the proliferation of MG63 cells was enhanced significantly (**Figure 1**, $P<0.001$). These results indicated that HA/SPEEK/PEEK composites promoted proliferation of osteoblast cell MG63.

10% HA/SPEEK/PEEK composite promoted the differentiation of MG63

MG63 cells were incubated with extracts of PEEK, SPEEK/PEEK, HA, and 10% HA/SPEEK/PEEK for 14 days, and then alizarin red staining was performed to detect the cell differentiation. As shown in **Figure 2**, cells in the HA group and 10% HA/SPEEK/PEEK group showed more red nodes. ALP and COLI are important markers of differentiation of osteoblast. MG63 cells were incubated with extracts of PEEK, SPEEK/PEEK, HA, and 10% HA/SPEEK/PEEK for 14 days, 21 days and 28 days, and then levels of ALP and COLI were measured by qRT-PCR. Results of qRT-PCR showed that the ALP level in cells incubated with extract of SPEEK/PEEK had no significant difference with the blank group, and ALP level in PEEK group was even lower than that of the blank group. Results of qRT-PCR also showed that ALP level in HA group was slightly higher than that of the blank group, but not significantly. In 10% HA/SPEEK/PEEK group, ALP level was increased significantly (**Figure 3A**). The most significant changes were discovered in the 28th day.

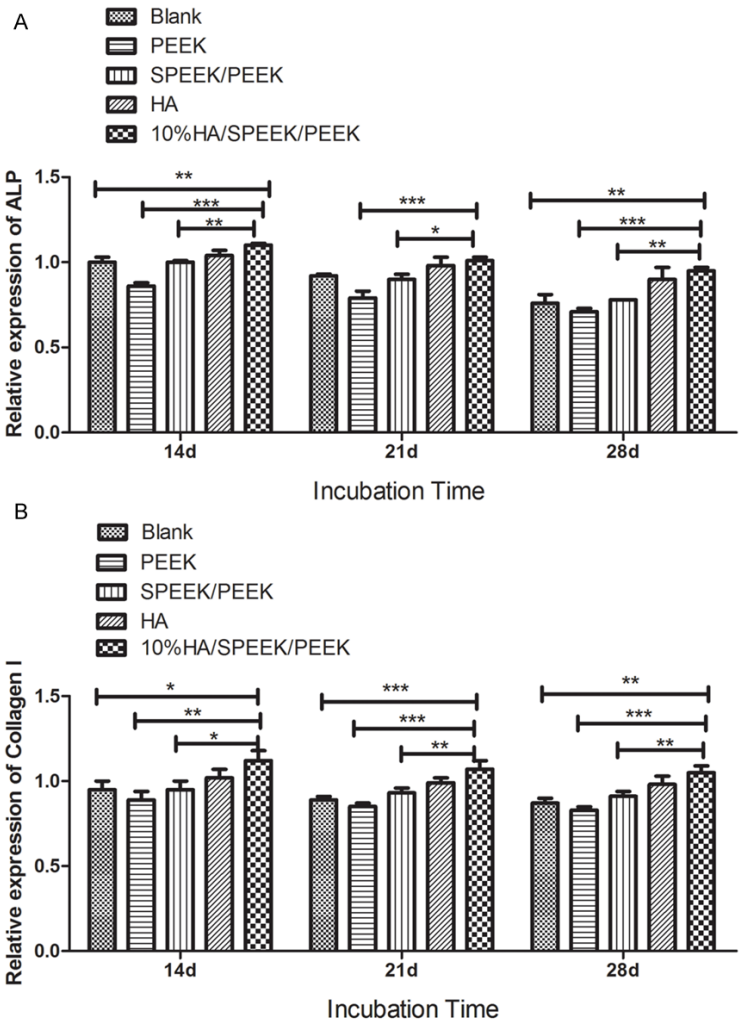

Figure 3. 10% HA/SPEEK/PEEK composite increased the mRNA levels of ALP and Collagen I. A. mRNA level of ALP in blank, PEEK, SPEEK/PEEK, HA, and 10% HA/SPEEK/PEEK groups was detected by qRT-PCR. Relative mRNA level was normalized to β-actin and calculated using the $2^{-\Delta\Delta Ct}$ method. B. mRNA level of Collagen I in each group was detected by qRT-PCR with β-actin as the internal reference. All experiments were repeated three times and the results are shown as mean ± SD. *P<0.05, **P<0.01, ***P<0.001.

Similar change pattern was also discovered in level of COLI. There was no significant changes in PEEK group, SPEEK/PEEK group, and HA group, however, COLI level in 10% HA/SPEEK/PEEK group was increased significantly (**Figure 3B**). These results suggested that 10% HA/SPEEK/PEEK promoted the differentiation of MG63.

10% HA/SPEEK/PEEK composite promoted the activation of ERK signaling pathway

To further explore the mechanism underlying the effects of 10% HA/SPEEK/PEEK composite, the activation of ERK signaling pathway was detected by western blot. As shown in **Figure 4**, protein levels of p-ERK in PEEK group and SPEEK/PEEK group showed no difference with those in the blank group. However, after treatment with HA extract, level of p-ERK was increased significantly (P<0.01), and level of p-ERK was even higher in 10% HA/SPEEK/PEEK group. There was no significant difference in the ERK levels in each group. These results indicated that the ERK signaling pathway was involved in the function of 10% HA/SPEEK/PEEK composite.

10% HA/SPEEK/PEEK composite promoted the activation of JNK signaling pathway

The activation of JNK signaling pathway was also detected in our study. As shown in **Figure 5**, level of p-JNK in PEEK group and SPEEK/PEEK group showed no difference compared with that of blank group. The level of p-JNK in HA group showed a slight increase, but there was no significant difference. Comparing with the blank group, the p-JNK level in the 10% HA/SPEEK/PEEK group showed a significant increase, with no changes in the JNK levels in each group. These results indicate that JNK signaling pathway was activated by 10% HA/SPEEK/PEEK composite.

10% HA/SPEEK/PEEK composite promoted the activation of P38 signaling pathway

The activation of P38 signaling pathway was detected by western blot. There were no changes in the protein level of P38. P-P38 level in PEEK group and SPEEK/PEEK group showed no significant changes compared with that in the blank group. However, after treatment with

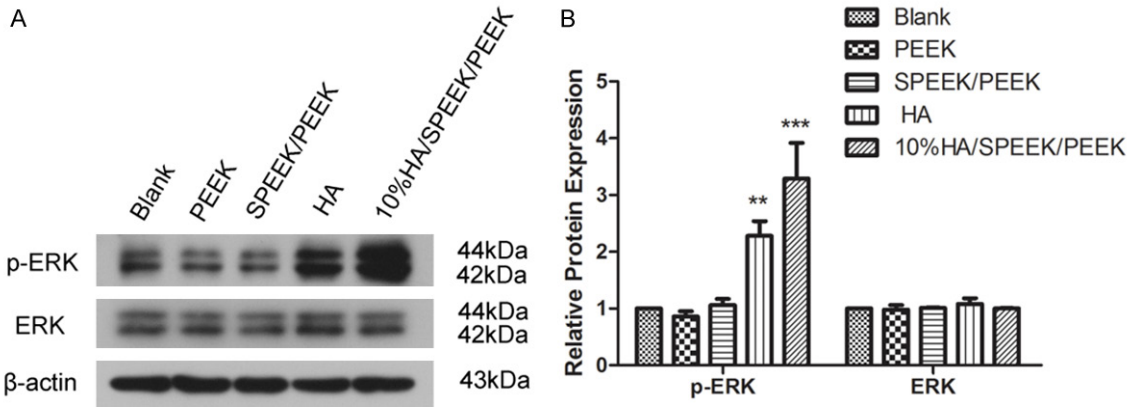

Figure 4. 10% HA/SPEEK/PEEK composite promoted the activation of ERK signaling pathway. A. The protein levels of ERK and p-ERK were detected by western blot using β-actin as the internal reference. B. Relative protein levels of ERK and p-ERK were calculated. All experiments were repeated three times and typical results are presented. The results are shown as mean ± SD. **P<0.01, ***P<0.001.

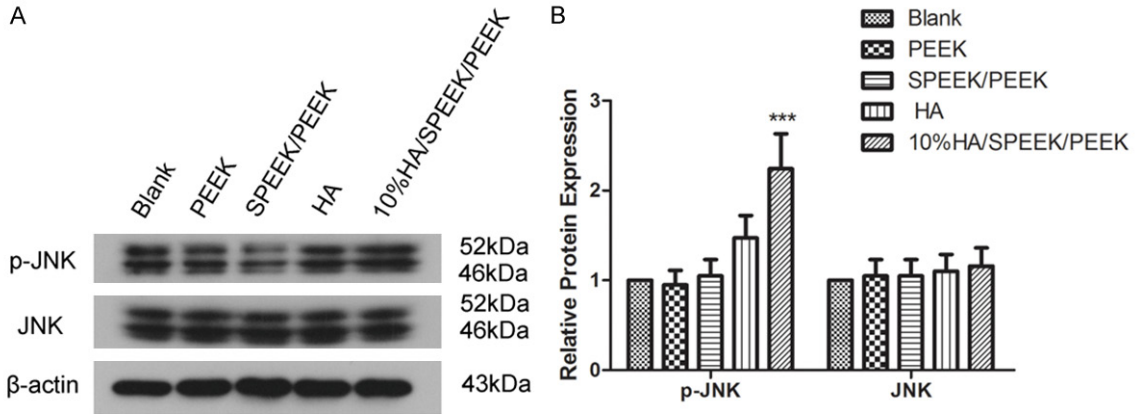

Figure 5. 10% HA/SPEEK/PEEK composite promoted the activation of JNK signaling pathway. A, B. Protein levels of JNK and p-JNK were detected by western blot and the relative protein levels were calculated using β-actin as the internal reference. Each experiment was repeated three times and typical results are presented. The results are shown as mean ± SD. ***P<0.001.

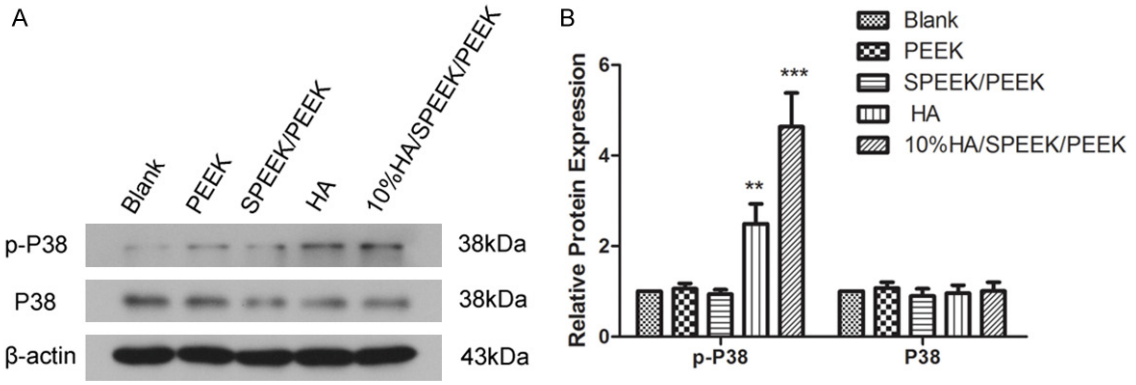

Figure 6. 10% HA/SPEEK/PEEK composite promoted the activation of P38 signaling pathway. A. The levels of P38 and p-P38 were detected by western blot with β-actin as the internal reference. B. Relative protein level was calculated. Typical results are shown. All experiments were repeated three times and the results are shown as mean ± SD. **P<0.01, ***P<0.001.

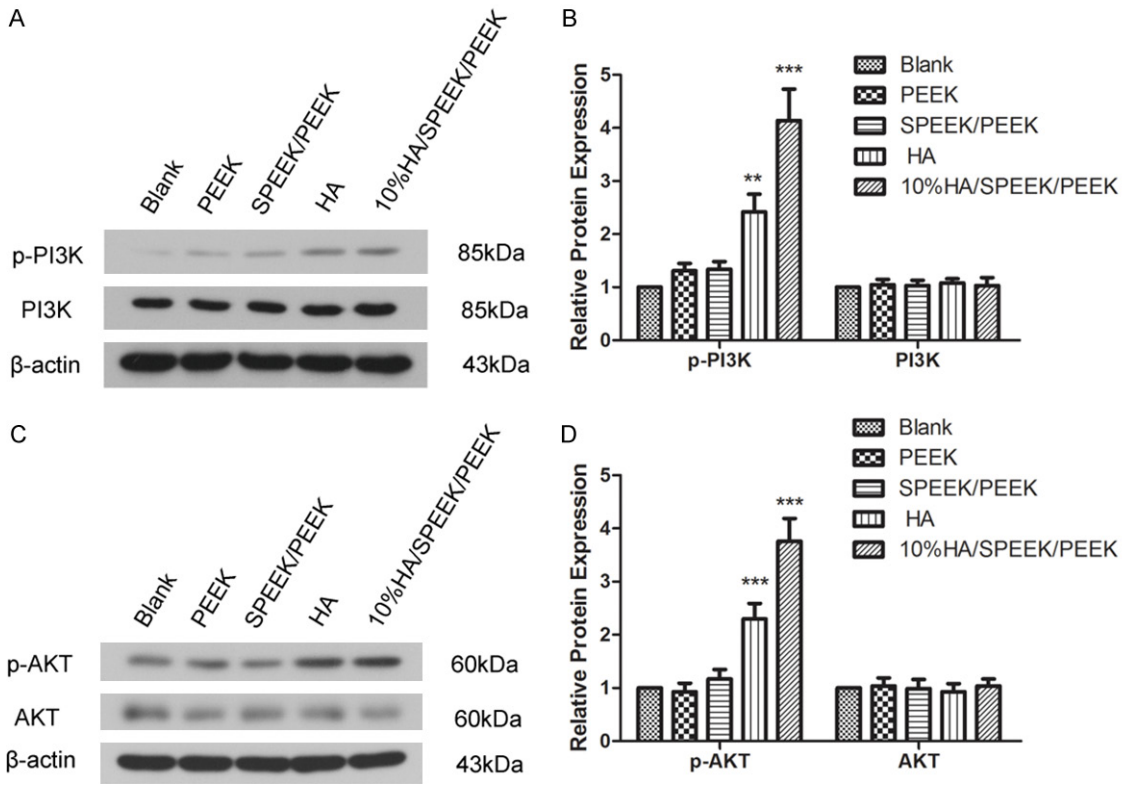

Figure 7. 10% HA/SPEEK/PEEK composite promoted the activation of PI3K/AKT signaling pathway. A, B. Protein levels of PI3K and p-PI3K were detected by western blot using β-actin as the internal reference, and the relative protein level was calculated. C, D. Levels of AKT and p-AKT were detected by western blot with β-actin as the internal reference. Each experiment was repeated three times, and the results are presented as mean ± SD. Typical results are presented. **P<0.01, ***P<0.001.

HA extract, the p-P38 level was increased significantly (**Figure 6**, P<0.01). P-P38 level in the 10% HA/SPEEK/PEEK group showed a more significant increase comparing with that in the blank group (P<0.001). These results suggested that the activation of P38 signaling pathway was also involved in the effects of 10% HA/SPEEK/PEEK.

10% HA/SPEEK/PEEK composite promoted the activation of PI3K/AKT signaling pathway

The PI3K/AKT signaling pathway plays important roles in the proliferation and differentiation of cells. And the activation of PI3K/AKT signaling pathway were explored in the present study. Results of our study showed that there were no changes in the protein levels of PI3K and AKT. The level of p-PI3K in PEEK group and SPEEK/PEEK group showed no difference with that in the blank group. However, after treatment with HA extract, the p-PI3K level was increased significantly (**Figure 7A** and **7B**, P<0.01), with a more significant increase in the 10% HA/SPEEK/PEEK group. The level of p-AKT was also increased by HA treatment and more significant changes were discovered in the 10% HA/SPEEK/PEEK group (**Figure 7C** and **7D**). These results revealed that the PI3K/AKT signaling pathway was involved in the function of 10% HA/SPEEK/PEEK composite.

Discussion

In the present study, function of a composite of HA and PEEK (10% HA/SPEEK/PEEK) was explored. 10% HA/SPEEK/PEEK composite was found to promote the proliferation and differentiation of osteoblast cells. Further mechanism study showed that MAPK and PI3K/AKT signaling pathways were involved in the function of 10% HA/SPEEK/PEEK composite.

In our study, effects of 10% HA/SPEEK/PEEK composite on the proliferation and differentia-

tion of osteoblast cells were explored. Results of our study showed that 10% HA/SPEEK/PEEK composite can promote the proliferation and differentiation of osteoblast cells. HA is the main active ingredient of the composite. Natural HA has the ability to promote the proliferation and osteogenic differentiation of MSCs [14]. Nano HA was also reported to promote the proliferation and adhesion of human periodontal ligament cells [15, 16]. However, the physical and chemical properties of HA are poor. PEEK shows suitable physical and chemical properties, but the bioactivity is low. Untreated PEEK is bioinert and its surface is hydrophobic and not osseointegrated [17]. Combining these two materials may result in a more suitable bone-implant material. In our study, 10% HA/SPEEK/PEEK composite showed an enhanced bioactivity. HA is regarded as an excellent coating material, Par Johansson and colleagues showed that nano HA coating promoted the biocompatibility and bioactivity of the PEEK and further accelerated osseointegration [18]. Nano HA coated PEEK showed an enhanced bone healing effect [19, 20]. Combining HA to PEEK, either mixture or applied to the surface of PEEK, enhanced the bioactivities of PEEK [12, 21].

The MAPK signaling pathway is involved in the early stage of osteoblast differentiation [22]. In our study, results showed that the ERK, JNK, and P38 signaling pathways were activated by 10% HA/SPEEK/PEEK composite. These results suggest that 10% HA/SPEEK/PEEK composite may promote the proliferation and differentiation of osteoblast cells through MAPK signaling pathway. Zhang and colleagues show that MAPK signaling pathway as well as other genes and microRNAs is involved in natural HA-induced osteogenic differentiation [14]. Reports of Song and colleagues showed that HA can also active the ERK signaling pathway in osteoblast cells [23]. Through the P38 signaling pathway, HA also increases BMP-2 expression level to promote differentiation of periodontal ligament cells [24]. Both HA and 10% HA/SPEEK/PEEK composite can active the ERK and P38 signaling pathways, which suggests that 10% HA/SPEEK/PEEK composite may perform its function in a similar way to that of HA.

The activation of PI3K/AKT signaling pathway is also associated with the osteogenic differentiation. In our study, PI3K/AKT signaling pathway was also activated by 10% HA/SPEEK/PEEK composite. Through PI3K/AKT signaling pathway, nano HA also affects proliferation of periodontal ligament cells [15, 25]. Consistent with our study, Woo and colleagues showed that, through that activation of AKT signaling pathway, scaffolds composed of poly(l-lactic acid) (PLLA) and HA resulted in less cell apoptosis compared with that of PLLA scaffolds [26]. Epidermal growth factor receptor (EGFR), which is the up-stream of ERK and AKT, was also involved in the function of nano HA [15].

Report of Chen and colleagues showed that, through the activation of MAPK signaling pathway, HA exposure caused decreased cell viability, increased cell apoptosis and necrosis and in hepatocytes, and inflammatory cell infiltration and oxidative stress in the liver, which will result in hepatotoxicity and liver injury [27]. These indicate that the use of 10% HA/SPEEK/PEEK composite may cause hepatotoxicity, but further studies are needed.

In our study, we explored the effects of 10% HA/SPEEK/PEEK composite and found that 10% HA/SPEEK/PEEK composite promoted the proliferation and differentiation of osteoblast cells. Further mechanism study indicated that the activation of MAPK signaling pathway and PI3K/AKT signaling pathway were involved in the function of 10% HA/SPEEK/PEEK composite. Our study suggests that 10% HA/SPEEK/PEEK composite may become a promising bone-implant material, but further explorations are still needed.

Acknowledgements

This study was supported by grants from the Science and Technology Project of Shenzhen (No.: JCYJ20140411094009912) and the Science and Technology Project of Nanshan District, Shenzhen (No.: 2014003).

Disclosure of conflict of interest

None.

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