Materials Science and Engineering C 29 (2009) 1829–1834

Contents lists available at ScienceDirect

Materials Science and Engineering C

journal homepage: www.elsevier.com/locate/msec

Gene expressions of Collagen type I, ALP and BMP-4 in osteo-inductive BCP implants show similar pattern to that of natural healing bones

Lanying Sun [a,b], Ling Wu [b], Chongyun Bao [a,b,*], Chunhua Fu [a], Xinlong Wang [c], Jinfeng Yao [a], Xingdong Zhang [c], Clemens A. van Blitterswijk [b]

[a] State Key Laboratory of Oral Diseases, Sichuan University, Chendu, 610041, China
[b] Department of Tissue Regeneration, Institute of Biomedical Technology, University of Twente, Enschede, 7500 AE, The Netherlands
[c] National Engineering Research Center for Biomaterials, Sichuan University, Chendu, 610065, China

ARTICLE INFO

Article history:
Received 25 November 2008
Received in revised form 14 January 2009
Accepted 14 February 2009
Available online 1 March 2009

Keywords:
Biphasic calcium phosphate ceramics
Osteo-inductive materials
In vivo tissue engineering
Real-time polymer chain reaction

ABSTRACT

Osteo-inductive materials give rise to ectopic bone formation in vivo either in muscles or in subcutaneous tissue. However, the underlying molecular mechanism is totally unclear. To investigate the expression pattern of bone related genes in osteo-inductive materials, we performed quantitative PCR (qPCR) to detect the expressions of type I collagen, alkaline phosphatase (ALP) and bone morphogenetic protein 4 (BMP-4) in biphasic calcium phosphate (BCP) ceramics implanted in dorsal muscle of dogs. Bone formation in mandibular alveolus defects served as controls showing the expression patterns of these genes in natural healing bones. Histological examinations were performed to show the bone formation in osteo-inductive BCP implants. Data of qPCR indicated that all tested genes had a similar expression pattern with two peaks during the bone formation either in BCP implants or natural healing bones. Type I collagen and ALP were expressed at lower levels with delayed peak in BCP implant than that in natural healing bone. Higher BMP-4 expression level was detected in BCP ceramic implant than in natural healing bone at all the time points. These results demonstrated that expression patterns of bone-related genes in the inductive bone formation are similar to that of natural healing bone formation. As these three genes are important parameters for osteoblast activity in bone formation, our data provide clue to uncover the molecular mechanism of bone formation in osteo-inductive materials.

© 2009 Elsevier B.V. All rights reserved.

1. Introduction

The repair of massive and segmental bone defects caused by tumor resection, trauma, inflammation and congenital abnormality is a great challenge to surgeons. At present, autografts, allografts, xenografts and alloplastic grafts are generally used for the repair of bone defect [1–3]. Although autograft is preferred for bone substitution, it has several disadvantages, including the shortage of supply of suitable bone, pain in collection, risk of hemorrhage, infection, nerve damage and cosmetic disability [2]. Allograft and xenograft, however, may carry the unforeseen risks of immunological reaction and disease transmission [1]. Seeking for novel bone substitutes to improve bone repair is necessary. Using in vitro expanded mesenchymal stem cells and synthetic scaffolding materials to produce synthetic bone substitutes comparable to autologous bone is promising and such an approach is theoretically possible to repair damaged bone. However, partially due to the nutrition problem it is difficult to produce large tissue-engineered bone in practice at the moment. Meanwhile current bone tissue engineering technique is expensive and is therefore far from the clinics. To overcome shortages of tissue-engineered bones, synthetic materials which possess bone inducing ability comparable to autografts based on osteo-inductive materials would be an alternative approach.

An osteo-inductive material gives inductive ectopic bone formation (either in muscles or subcutaneous tissue) [4,5]. Inductive bone formation was observed in various calcium phosphate ceramics including coral-derived or synthetic hydroxyapatite ceramics, tricalcium phosphate ceramics, calcium phosphate cement, and biphasic calcium phosphate ceramics in pigs, goats, dogs, monkeys, baboons and even in humans [6,7]. It is assumed that osteo-inductive synthetic materials concentrate growth factors from surrounding body fluids and induce mesenchymal stem cells attracted to form bone. Therefore, it is possible to use osteo-inductive synthetic materials and body's own stem cells in soft tissues to produce “autologous” bone (in vivo tissue engineering bone) for bone repair [8,9].

Among various calcium phosphate ceramics, BCP ceramics (containing both HA and TCP) has been widely evaluated regarding inductive bone formation, because a reasonable solubility could be obtained via controlling Ca/P ratio and inducing bone easily formed with BCP [5,7]. Inductive bone formation in BCP ceramics has been

* Corresponding author. State Key Laboratory of Oral Diseases, Sichuan University, Chendu, 610041, China. Tel.: +86 28 85501233; fax: +86 28 85582167.
E-mail address: cybao9933@scu.edu.cn (C. Bao).

0928-4931/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.msec.2009.02.011

shown to be morphologically and histologically similar to mature bone [10,11], while little information showing bone related gene expression in inductive bone formation.

Genetic studies and cell biology research revealed several important genes in osteoblasts during bone formation. Type I collagen, constituting over 90% of bone protein, serves as the template of bone matrix and controls the nucleation site and growth space of hydroxyapatite [12,13]. During bone formation, ostoeblasts interact with the environment, producing the organic matrix of collegens which are then deposited by calcium phosphate [13]. Alkaline phosphatase (ALP) is an important osteoblastic differentiation marker [14,15]. It reduces phosphate-containing substances to produce free phosphate for the requirement of bone mineralization [16,17]. ALP is associated with Pi homeostasis, and can hydrolyse a variety of phosphate compounds. By hydrolyzing pyrophosphate (PPi), a known inhibitor of HA formation, ALP regulates the mineralization process [16]. BMP-4 is one of the main contributing factors in new bone formation during the early phase of fracture healing and can improve bone regeneration [18–21]. BMP4 could act synergistically with VEGF to increase recruitment of mesenchymal stem cells in the early stages of endochondral bone formation, therefore, significantly enhance bone formation and bone healing [22]. Over-expression of BMP-4 by a retrovirus system induced muscle derived stem cells to differentiate into osteogenic lineage and improve bone healing in nude mice [23]. In bone formation, bone-related gene expression is strictly regulated, inappropriate gene expression might cause bone diseases [24–26]. Measuring the expression of these of bone-related genes in the inductive forming bone with osteo-inductive BCP ceramics may demonstrate the activity of osteoblasts at a molecular level. Furthermore, these data would be useful to help us to evaluate osteo-inductive syntheses in the osteo-inductive materials.

In this study, we performed qPCR to evaluate bone-related gene expression in osteo-inductive BCP ceramics implanted in muscle of. Type I collagen, ALP and BMP-4 were selected as target genes and their expressions in natural healing bone served as control.

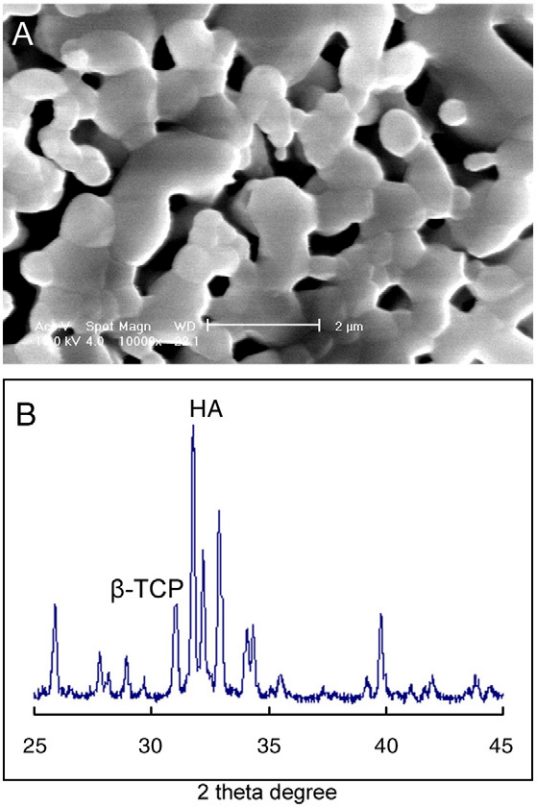

Fig. 1. SEM photographs of BCP ceramics. (A): Microstructure of BCP ceramics; (B): XRD photograph of BCP ceramics. HA and β-TCP phases were detected in the ceramics.

2. Materials and methods

2.1. Material preparation

Porous BCP ceramics with microporous structures on their macropore surface was prepared by foaming apatite powder with H_2O_2 and sintering at 1100 °C for 3 h (Fig. 1A). The porosity of the ceramics measured by Archimedes method was 60% and the pore sizes ranged from 300–400 μm. Chemical composition containing 60 wt.% HA and 40 wt.% β-TCP was analyzed with XRD (Fig. 1B). Ceramic cylinders with 5 mm in diameter and 8 mm in length were machined and then cleaned by ultrasonication in de-ionized water and autoclaved.

2.2. Animal surgery and morphological observation of samples

Fourteen adult dogs were used in this experiment in line with the International Guiding Principles for Animal Research (1985). After anaesthesia with 3% sodium pentobarbital, eight BCP ceramic cylinders were implanted into the dorsal muscles of each dog. Meanwhile, bone defect (5×5×5 mm) was created at mandibular alveolus to show the natural bone healing process. Following surgery, the locomotion and eating habits of the animals were normal. Animals were sacrificed by intravenous injection of overdose 3% sodium pentobarbital at 3 days, 1, 2, 4, 8, 12 and 24 weeks post-implantation respectively. At each time point, 2 specimens of BCP implants were harvested for histological analysis. The other 6 specimens of BCP implants and 2 samples of the bone from bone defect (natural healing bones) were snap-frozen in liquid nitrogen and stored at −150 °C until RNA isolation.

2.3. Histological analysis

For histological analysis, BCP implants were fixed, and then treated in decalcified solution (50% formic acid + 20% sodium citrate, 1:1) for 4 weeks. Decalcified samples then underwent procedures for dehydration, embedding, and incising. To evaluate the bone formation, hematoxylin and eosin staining (H&E) and Masson's trichrome method were performed.

2.4. Total RNA extraction

All frozen specimens were pulverized with mortar in liquid nitrogen. The specimens being in the dorsal muscles for 24 weeks and natural bone healing were broken into pieces before being pulverized. The pulverized specimens was added to TRIzol reagent (Invitrogen, Carlsbad, CA, USA), homogenized and the total RNA was purified by following the manufacturer's instructions. Firstly, 1 ml of the homogenized solution containing RNA was collected, mixed with 0.2 ml of chloroform, and centrifuged at 12,000 g for 15 min at 4 °C. Thereafter RNA in aqueous phase (top) was transferred into a fresh tube, precipitated by mixing 0.5 ml of isopropyl alcohol and recovered by centrifuging the tube at 12,000 ×*g* for 10 min at 4 °C. Then the RNA pellet was washed in 1 ml of 75% ethanol and centrifuged at 7500 ×*g* for 5 min at 4 °C. Finally, the whole RNA pellet was dissolved in 100 μl of diethylpyrocarbonate water and its quantity was assessed using a Smartapec™ Plus spectrophotometer (BIO-RAD, USA) by measuring absorption ratios at 260/280 nm. After the integrity of RNA was confirmed by agarose gel electrophoresis, the RNA was immediately frozen at −70 °C until reverse transcription.

2.5. Reverse transcription

All RNA samples having an $A_{260/280}$ ratio > 1.9 were used in reverse transcription. cDNA was synthesized by reverse transcription on a

Table 1
Primer sequences, theoretical melt temperature (Tm), length, product size and GenBank accession number used in quantitative PCR.

Gene name	Primer sequence	Tm (°C)	Len (bp)	Product size (bp)	Accession no.
GAPDH	F: 5′-ggtgctgagtatgttgtggagtc-3′	60.2	23	174	AB_038240
	R: 5′-gctgacaatcttgagggagttg-3′	58.1	22		
Collagen-I	F: 5′-aagaaccccaaggagaagagac-3′	58.1	22	168	NM_001003090
	R: 5′-cttgcagtggtaggtgatgttc-3′	58.1	22		
ALP	F: 5′-gagatggacaagttcccttacg-3′	58.1	22	177	XM_535374
	R: 5′-ctcgtttccctgagtcgtgtt-3′	57.8	21		
BMP-4	F: 5′- gggcttccaccgaataaaca -3′	55.4	20	142	XM_859035
	R: 5′-cgcagggctcacatcaaa-3′	54.3	18		

Mastercycler gradient instrument (Eppendorf, Germany) with RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Lithuania) under the following conditions: 70 °C for 5 min, 37 °C for 5 min, 42 °C for 60 min, and stopped at 70 °C for 10 min. The first strand cDNA obtained was stored at −20 °C.

2.6. Quantitative real-time PCR

Real-time PCR based on SYBR Green I methodology were performed to detect a set of bone-related genes, type I collagen, ALP, BMP-4 and GAPDH as endogenous control. The PCR efficiencies for all tested gene were ensured to be close to 1 by optimizing the reaction condition. The relative quantitative real-time PCR was run on samples ($n=3$) using ABI Prism® 7300 Real-Time PCR System instrument (Applied Biosystems, USA). Reaction in a volume of 25 µl contained 12.5 µl of 2× SYBR® Premix Ex Taq™ (TaKaRa, Japan), 0.5 µl of forward primer (10 µM), 0.5 µl of reverse primer (10 µM), 0.5 µl of ROX Reference Dye (50×), 2.0 µl of cDNA and 9 µl of dH_2O. PCR + dissociation routine: 95 °C for 10 s, 40 cycles of 95 °C for 5 s and 60 °C for 31 s, 95 °C for 15 s, 60 °C for 30 s and 95 °C for 15 s (Dissociation phase in italics). The oligonucleotides sequences of the PCR primer pairs that were used for each gene were listed in Table 1.

The cycle threshold (C_T) values provided from the report of real-time PCR were imported into Microsoft Excel. For each sample, the relative amounts of target genes normalized by GAPDH were calculated by $2^{-\Delta CT}$ method where $\Delta C_T = C_{T,Target} - C_{T,\ GAPDH}$ [27].

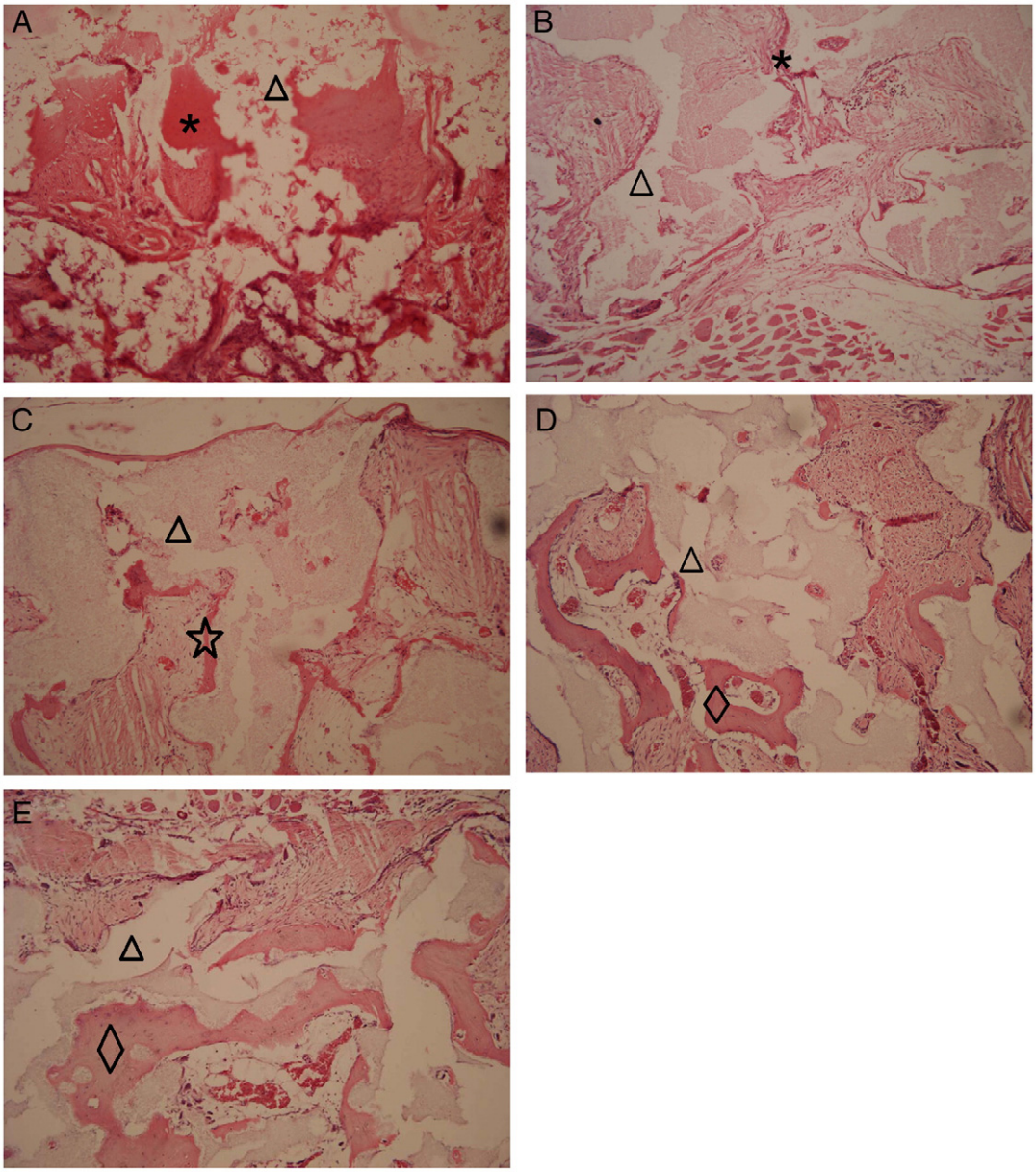

Fig. 2. H&E staining of decalcified sections of ceramic implants at different time points: 1-week (A), 2-week (B), 4-week (C), 12-week (D) and 24-week (E); triangles represent ceramics, asterisks represent bone-like tissue, pentacles represent trabicular bone and rhombus represent mature bone. Bar = 100 µm.

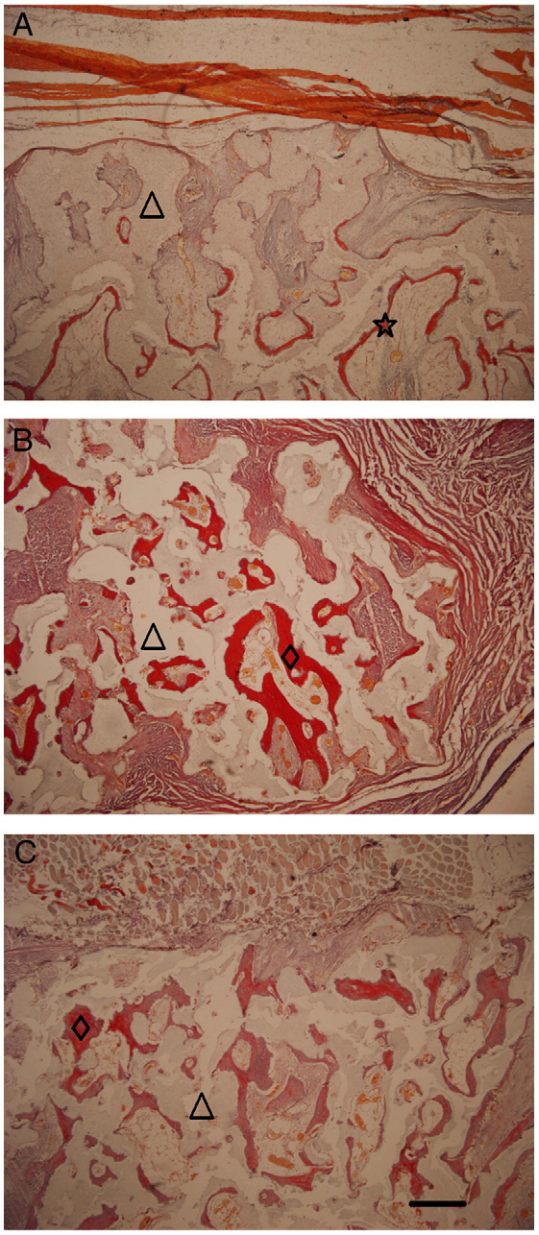

Fig. 3. Masson's trichrome staining of decalcified sections of ceramic implants at different time points: 4-week (A), 12-week (B) and 24-week (C). Bar = 100 μm.

2.7. Statistic analysis

Analysis of variance (ANOVA) (SPSS, version 13.0) was performed for each target gene to evaluate the significant difference of gene expression over time and between BCP and controls. $P<0.05$ was considered significant.

3. Results

3.1. Histological analysis of bone formation in BCP ceramic implants

BCP cylinders were encapsulated by normal muscle tissues after 1-week implantation in muscle of dog and became more tenacious with time up to 24 weeks, while specimens harvested at 3-days were brittle and did not connect with muscles tightly. None of the implanted HA/TCP ceramics was fully degraded during the time period up to 24 weeks. H&E staining and Masson's trichrome method showed that gradual bone formation can be found in the ceramic implants at different time points. At 3-day, most of the cells were fibroblasts and few osteoblasts were observed. Bone-like tissue could be observed at 1-week and 2-week (Fig. 2A and B), and trabecular bone was found at 4-week implants (Figs. 2C and 3A). In some regions of the pore, osteoblast differentiation occurred directly aggregated at the inner surface. Mature bone was observed in the pores of ceramics at 12-week and 24-week implant (Figs. 2D, E,3B and C). Woven bone with osteocytes was seen on the pore surface of BCP ceramic. No bone like tissues could be observed outside the implants.

3.2. Gene expression in natural healing bone

A similar pattern was found in the expression of Type I collagen, ALP and BMP-4. The expression levels were initially increased to the first peak at week 1 and then dropped to the lowest level at week 4, whereas increased slightly to reach the second peak at week 12. Apparently, the second peak at week 12 is not comparable to the first one at week 1 (Fig. 4). This expression pattern of bone-related genes in natural healing

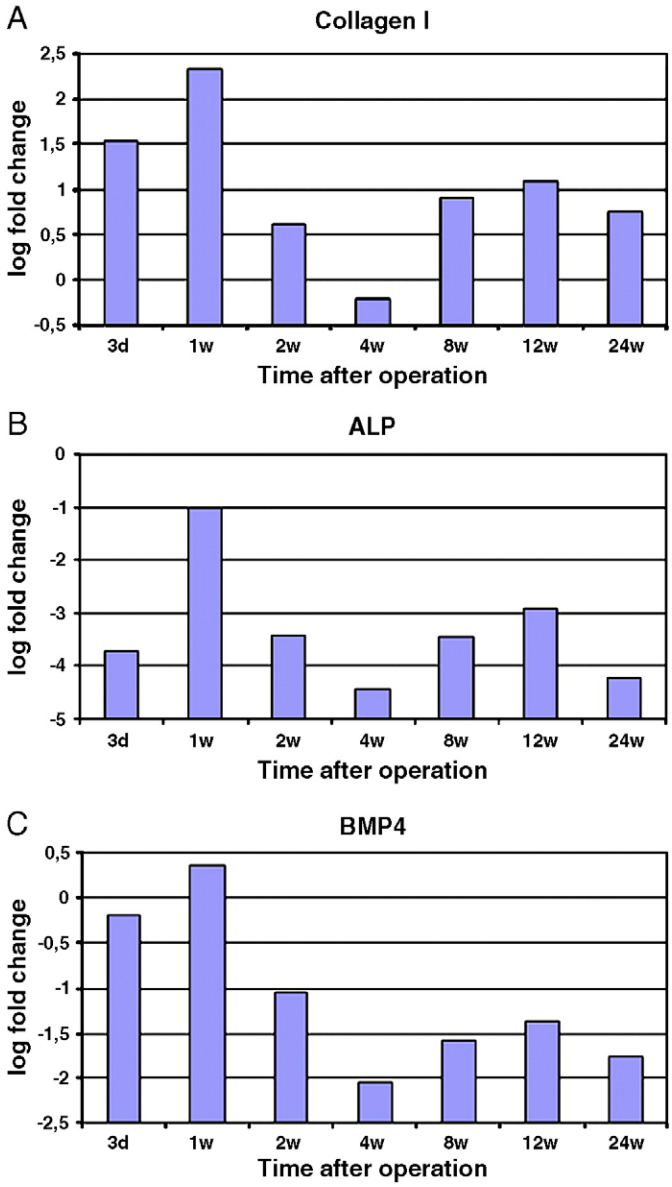

Fig. 4. Relative quantitative real-time PCR expression in natural healing bone of type I collage (A), ALP (B), and BMP-4 (C). Data were normalized to GAPDH. In order to highlight the differences in different ranges of expression, a logarithm scale was used. d stands for day and w stands for week.

bones indicated that week 1 and week 12 may be the crucial time points for osteogenic differentiation and bone formation.

3.3. Gene expression in BCP implant

All tested genes, including Type I collagen, ALP and BMP-4 were detectable in BCP ceramics implants during the experimental period. The expression pattern of type I collagen and ALP in BCP ceramics implants showed some differences when compared with natural healing bones. Type I collagen mRNA expression increased steadily from day 3 to a peak at week 12 followed by a considerable down-regulation at week 24 (Fig. 5A). ALP mRNA had the same pattern to type I collagen, but the maximum expression was at week 8 (Fig. 5B). BMP-4 showed completely equal expression pattern to natural healing bone: first peak was at week 1, lowest was at week 4 and second peak was at week 12 (Fig. 5C).

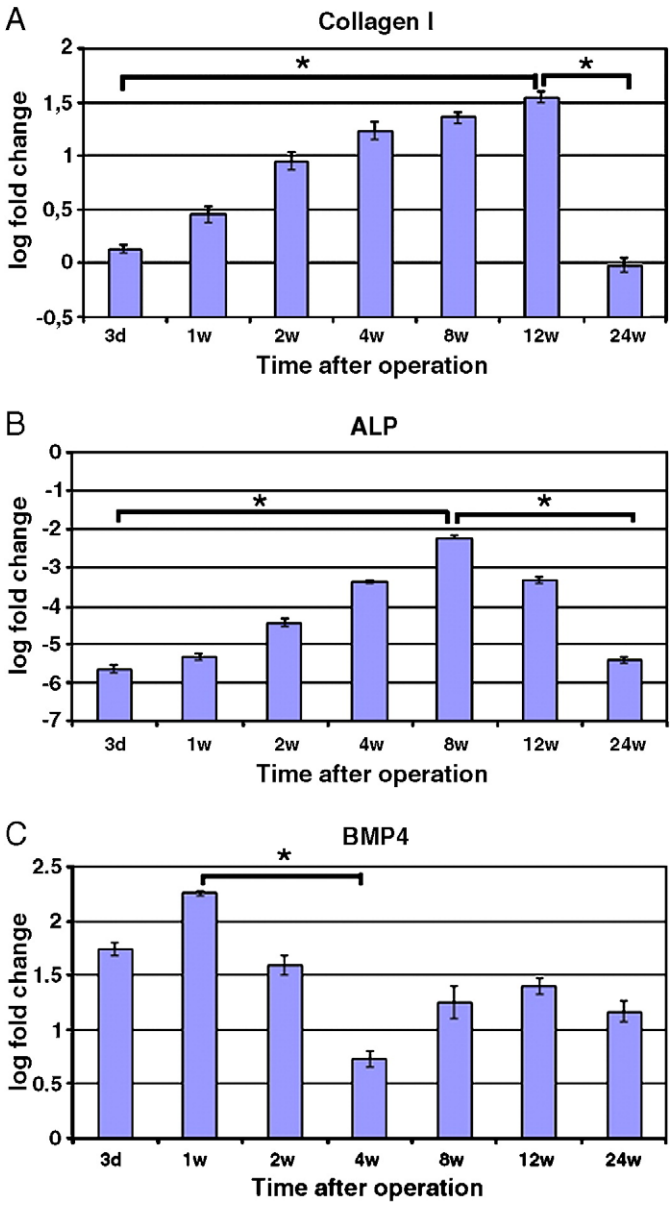

Fig. 5. Relative quantitative real-time PCR expression in BCP ceramics of type I collage (A), ALP (B), and BMP-4 (C). Data were normalized to GAPDH and represented as mean ± S.D. In order to highlight the differences in different ranges of expression, a logarithm scale was used. d stands for day and w stands for week. Asterisk shows significant difference ($p<0.01$).

4. Discussion

In this study, we investigated the expression pattern of type I collagen, ALP and BMP-4 in BCP ceramics implants and natural healing bones. Despite some differences on expression level, expression of tested genes in BCP ceramics implants and natural healing bones shared a similar expression tendency which was initially up-regulated and finally down-regulated.

Calcium phosphate ceramics are widely used as bone substitute materials because of their similarity to the mineral phase of bone, absence of antigenicity, and excellent osteoconductivity [28]. Previous studies have demonstrated that calcium phosphate ceramics with special chemical constituent and physical structure could induce bone formation in soft tissue [4,7]. The bone produced with osteo-inductive calcium phosphate ceramics in soft tissues had similar histological characteristic to natural bone and did not disappear nor over-grow in 2.5 years [29]. In this study, we examined bone formation in BCP ceramics implants with H&E staining and Masson's trichrome method. Our results were in agreement with the previous reports that bone formation could be found in BCP implants. At the early stage (1 week), only bone-like tissue was observed in the ceramic implants. Then gradually (form 2 week to 4 week), bone tissues grew into the pores of ceramics forming trabicular bones in the implants. Finally (for 12 week to 24 week), bone like tissues turned out to be mature bones.

Bone formation is an intricate and ordered cascade of synthesis of matrix proteins and calcium phosphate in a continuously renewed biological environment and regulated by a cluster of growth factors [30–32]. In bone formation, bone-related genes are strictly regulated to ensure correct chronological order and each gene has a unique expression profile [33]. Here, we employed mandibular alveolus defects to characterize the gene expression profile in nature healing bones. It's interesting that all tested genes including Collagen type I, ALP and BMP-4 showed the same expression pattern with two peaks at week 1 and week 12 and one trough at week 4. Although there were piles of literatures describing the molecular aspects of bone healing, however, none had ever reported such a detailed expression pattern of Collagen type I, ALP and BMP-4 in the process of natural healing bones [34–36]. And our results also indicated that week 1 and week 12 may be the crucial time points for osteogenic differentiation and bone formation. Anyway, the data represented here provided us standards of gene expression in bone formation witch are vital for evaluating the gene expression in BCP implants.

Then, we performed qPCR to examine the expression patterns of Collagen type I, ALP and BMP-4 in BCP implants. Our data showed that all the three genes were continuously expressed in BCP ceramics, but at a different level when compared to the natural healing bones. It was reported that both Collagen type I and ALP are highly expressed in the early stage of bone formation [37]. In this study, however, expression of Collagen type I and ALP increased slowly with later peaks at week 12 and week 8, respectively. This may be due to fewer osteoblasts in the BCP ceramic implant at early stage. With the immigration and proliferation of osteoblasts in the BCP ceramic implants, the gene expressions of the type I collagen and ALP in BCP ceramics became considerable at later stage. Additionally, the maximum expression level of type I collagen and ALP mRNA seemed to be lower in BCP ceramics implants, suggesting the newly formed bone tissues in BCP ceramics implants not as many as that in natural healing bones. Although peaked at different time points, expression of type I collagen and ALP in BCP ceramic implants shared a common tendency: initially up-regulated and finally down-regulated. From this point of view, expressions of Collagen type I and ALP in BCP ceramics implants also shared a similar pattern with that in natural healing bones.

The most interesting finding in this study is that the expression pattern of BMP-4 in BCP ceramics implants is the same as that in natural healing bones. As a multifunctional growth factor belonging to the TGF-β super family, BMP-4 is known to play an important role in

new bone formation during the early phase of fracture healing and can improve bone regeneration [21,31]. Over-expression of BMP-4 has been demonstrated to improve bone formation in vivo [19,23]. Studies have shown muscle-derived stem cells (MDSCs) undergo osteogenic differentiation in response to BMP-4 in a dose dependant manner [38]. In the present study, it is possible that BMP-4 was expressed to act as stimulating factor for MDSCs surrounding the BCP ceramics implants. This hypothesis was supported by the fact that: events stimulated by BMP-4 began with stem cells chemotaxis, proliferation and expressing more BMP-4, followed by their subsequent differentiation into bone-forming cells [39–41]. So, it showed the same pattern with natural healing bones. However, fewer cells in the BCP ceramics implants delayed the expression of Collagen type I and ALP, resulting a later peak of expression at week 12 and week 8. Moreover, this also explained that more BMP-4 was expressed in BCP ceramics than in natural healing bone at all time points, because more BMP-4 would be needed to initiate the bone healing cascade in osteo-inductive materials.

5. Conclusions

Our results demonstrated that bone formation could be induced in non-osseous site with porous HA/TCP ceramics. Expression pattern of Collagen type I, ALP and BMP-4 indicated that inductive bone formation in BCP ceramics implants was later than that in natural healing bones. Despite some differences of expression level and peak time, the expression of these genes shared a similar pattern of initially up-regulated and finally down-regulated both in BCP ceramics implants and natural healing bones.

Acknowledgments

The authors appreciated Dr. Huipin Yuan of Twente University/ Progentix BV, the Netherlands, for his suggestion on manuscript preparation. This work was supported by the Natural Science Foundation of China (30672337) and Chinese Ministry of Education (NCET 06-0782).

References

[1] H. Burchardt, Clin. Orthop. Relat. Res. (1983) 28.
[2] B. Lu, X. Lu, Z. Zhang, J. Zhang, H. Bu, J. Cheng, Y. Li, Key Eng. Mater. 288–289 (2005) 237.
[3] T. von Arx, D.L. Cochran, R.K. Schenk, D. Buser, Int. J. Oral Maxillofac. Surg. 31 (2002) 190.
[4] H. Yuan, J.D. De Bruijn, Y. Li, J. Feng, Z. Yang, K. De Groot, X. Zhang, J. Mater. Sci., Mater. Med. 12 (2001) 7.
[5] H. Yuan, K. Kurashina, J.D. de Bruijn, Y. Li, K. de Groot, X. Zhang, Biomaterials 20 (1999) 1799.
[6] U. Ripamonti, J. Bone Jt. Surg. Am. 73 (1991) 692.
[7] H. Yuan, Z. Yang, J.D. De Bruij, K. De Groot, X. Zhang, Biomaterials 22 (2001) 2617.
[8] C. Bao, H. Fan, C. Deng, y. CaO, Y. Tan, Q. Zhang, X. Zhang, Key Eng. Mater. 254–256 (2004) 801.
[9] R.F. Service, Science 309 (2005) 683.
[10] K. Kurashina, H. Kurita, Q. Wu, A. Ohtsuka, H. Kobayashi, Biomaterials 23 (2002) 407.
[11] Z.J. Yang, H. Yuan, P. Zou, W. Tong, S. Qu, X.D. Zhang, J. Mater. Sci. Mater. Med. 8 (1997) 697.
[12] M.M. Giraud Guille, G. Mosser, C. Helary, D. Eglin, Micron 36 (2005) 602.
[13] M. Tzaphlidou, Micron 36 (2005) 593.
[14] M. Akahane, A. Nakamura, H. Ohgushi, H. Shigematsu, Y. Dohi, Y. Takakura, J. Tissue Eng. Regen. Med. 2 (2008) 196.
[15] H. Qu, M. Wei, Acta Biomater. 2 (2006) 113.
[16] M. Balcerzak, E. Hamade, L. Zhang, S. Pikula, G. Azzar, J. Radisson, J. Bandorowicz-Pikula, R. Buchet, Acta Biochim. Pol. 50 (2003) 1019.
[17] A.L. Williford, L.M. Pare, G.T. Carlson, Neonatal Netw. 27 (2008) 57.
[18] Y. Chen, K.M. Cheung, H.F. Kung, J.C. Leong, W.W. Lu, K.D. Luk, Biochem. Biophys. Res. Commun. 298 (2002) 121.
[19] K.D. Luk, Y. Chen, K.M. Cheung, H.F. Kung, W.W. Lu, J.C. Leong, Biochem. Biophys. Res. Commun. 308 (2003) 636.
[20] H. Peng, A. Usas, B. Gearhart, A. Olshanski, H.C. Shen, J. Huard, J. Bone Miner. Res. 19 (2004) 630.
[21] T. Sakou, Bone 22 (1998) 591.
[22] H. Peng, V. Wright, A. Usas, B. Gearhart, H.C. Shen, J. Cummins, J. Huard, J. Clin. Invest. 110 (2002) 751.
[23] V. Wright, H. Peng, A. Usas, B. Young, B. Gearhart, J. Cummins, J. Huard, Mol. Ther. 6 (2002) 169.
[24] D.M. Lawton, J.G. Andrew, D.R. Marsh, J.A. Hoyland, A.J. Freemont, Mol. Pathol. 52 (1999) 92.
[25] J. Rantakokko, H. Uusitalo, T. Jamsa, J. Tuukkanen, H.T. Aro, E. Vuorio, J. Bone Miner. Res. 14 (1999) 1934.
[26] F.H. Wezeman, M.A. Emanuele, N.V. Emanuele, S.F. Moskal II, M. Woods, M. Suri, J. Steiner, N. LaPaglia, Alcohol., Clin. Exp. Res. 23 (1999) 1534.
[27] K.J. Livak, T.D. Schmittgen, Methods 25 (2001) 402.
[28] T. Guda, M. Appleford, S. Oh, J.L. Ong, Curr. Top. Med. Chem. 8 (2008) 290.
[29] S.X. Qu, X. Guo, J. Weng, J.C. Cheng, B. Feng, H.Y. Yeung, X.D. Zhang, Biomaterials 25 (2004) 659.
[30] Z.S. Ai-Aql, A.S. Alagl, D.T. Graves, L.C. Gerstenfeld, T.A. Einhorn, J. Dent. Res. 87 (2008) 107.
[31] U. Ripamonti, M. Heliotis, C. Ferretti, Oral Maxillofac. Surg. Clin. North Am. 19 (2007) 575.
[32] U.M. Wikesjo, Y.H. Huang, G. Polimeni, M. Qahash, Oral Maxillofac. Surg. Clin. North Am. 19 (2007) 535.
[33] H. Bahar, D. Benayahu, A. Yaffe, I. Binderman, Crit. Rev. Eukaryot. Gene Expr. 17 (2007) 87.
[34] M. Hadjiargyrou, F. Lombardo, S. Zhao, W. Ahrens, J. Joo, H. Ahn, M. Jurman, D.W. White, C.T. Rubin, J. Biol. Chem. 277 (2002) 30177.
[35] E. Tsiridis, P.V. Giannoudis, Injury 37 (Suppl 1) (2006) S13.
[36] Z. Xiang, Y. Yang, X. Ma, W. Ding, Curr. Opin. Drug Discov. Dev. 6 (2003) 384.
[37] C. Wang, Y. Duan, B. Markovic, J. Barbara, C.R. Howlett, X. Zhang, H. Zreiqat, Biomaterials 25 (2004) 2507.
[38] K.A. Corsi, J.B. Pollett, J.A. Phillippi, A. Usas, G. Li, J. Huard, J. Bone Miner. Res. 22 (2007) 1592.
[39] M. Lind, Acta Orthop. Scand., Suppl. 283 (1998) 2.
[40] N.S. Cunningham, V. Paralkar, A.H. Reddi, Proc. Natl. Acad. Sci. U. S. A. 89 (1992) 11740.
[41] G. Li, H. Peng, K. Corsi, A. Usas, A. Olshanski, J. Huard, J. Bone Miner. Res. 20 (2005) 1611.