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Human bone marrow-derived mesenchymal stem cells and osteoblast differentiation on titanium with surface-grafted chitosan and immobilized bone morphogenetic protein-2

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Abstract Circulating progenitor cells are known to home to various organs to repair injured tissues or to routinely replace old cells and maintain tissue integrity. Similarly, circulating progenitor bone cells can possibly home to a bone implant, differentiate, and eventually osteointegrate with the prosthesis. Osteointegration of bone cells with the prosthesis can help to reduce the risk of implant failure due to constant movement between bone tissue and implant surface. In this study, we aim to investigate if immobilized bone morphogenetic protein-2 (BMP2) on chitosan-grafted titanium substrate (Ti-CS-BMP2) will enhance bone marrow-derived mesenchymal stem cell (BMMSC) adhesion onto the substrate surface and further induce their differentiation into osteoblasts. The results show that our Ti-CS-BMP2 substrate is able to retain adsorbed BMP2, and is capable of slow release of this growth factor. Despite the lesser number of BMMSCs initially attached onto the Ti-CS-BMP2 substrates and consequently the lower level of cell proliferation, Ti-CS-BMP2 cells had the highest level of ALP activity. RT-PCR results show that Ti-CS-BMP2 cells had a relatively higher level of transcription activity of Runx2, compared with that of bone cell-derived osteoblasts (BC-OB), an indication that the BMMSCs were actively differentiating into osteoblasts. Finally, alizarin red staining reveals the presence of calcium deposits in the differentiated cells. Hence our Ti-CS-BMP2 substrates possess an osteoconductive effect and can possibly be used to fabricate bone implants that can osteointegrate with host bone tissue.

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1 Introduction

Circulating mesenchymal stem cells (MSC) are known to migrate and home to various organs to mediate tissue repair [1, 2]. Similarly, it is possible that circulating bone progenitor cells can home to a bone implant and differentiate into bone-forming cells, eventually leading to osteointegration with the prosthesis. However, bacterial infection following surgical implantation of biomaterials and devices poses a risk to an implant. We have shown that titanium (Ti) with surface-grafted chitosan (CS) prevents bacterial adhesion, the mechanism of which is proposed to be due to the interaction between the oppositely charged bacterial cell wall and the CS [3, 4]. This interaction results in a change in bacterial membrane permeability, leading to the fatal leakage of cellular contents. Previous work have shown that BMP2-adsorbed titanium porous oxide implant surfaces had an osteoconductive effect which was surface- and dose-dependent [5]. The incorporation of BMP2 into biomimetic coatings like calcium phosphate has been shown to result in the formation of bone cells at the implant site [4, 5]. However, others have also reported that BMP2 immobilized onto implants does not increase bone formation [6]. In this study, we aim to investigate if BMP2 immobilized onto our bacteria-resistant chitosan-grafted titanium substrate will enhance BMMSC adhesion on the substrate surface and further induce their differentiation into osteoblasts.

2 Materials and methods

2.1 Materials

Materials and substrate preparation have previously been described [4]. Briefly, Ti–6Al–4V (subsequently denoted as Ti) foils were purchased from Goodfellow of Cambridge (UK). CS was purchased from CarboMer (San Diego, US) and refined twice by dissolving it in dilute acetic acid solution, filtered, then precipitated with aqueous sodium hydroxide and dried in a vacuum oven for 24 h at 40°C. The viscosity-average molecular weight was approximately 2.2×10^5 as determined by the viscometric method. The degree of deacetylation was 84% as determined by elemental analysis using the Perkin-Elmer Model 2400 elemental analyzer [7]. 3,4-Dihydroxyphenylalanine (dopamine) and glutaraldehyde were obtained from Sigma–Aldrich Chemical Co. (Singapore). Ultrapure water (>18.2 MΩ cm, Millipore Milli-Q system, Singapore) was used in the experiments. All materials used in cell culture were purchased from Gibco, Invitrogen (Singapore). Flow cytometry was done using the CyAn^{TM} ADP Analyzer. Fluoroisothiocynate (FITC)-conjugated CD44 and phycoerythrin (PE)-conjugated CD105 antibodies were purchased from eBioscience (San Diego, US), CD271-FITC was purchased from Miltenyi Biotec (GmbH, Germany), and CD34-FITC and CD45-FITC were purchased from AbD Serotec (UK). BMP2 was purchased from USBiological (US). The intensity and stability of BMP2 adsorbed on chitosan-grafted titanium substrate surface were assessed using the Bio-Rad DC Protein Assay (US). RNA and protein concentrations were measured using the NanoDrop ND1000 spectrophotometer. RT-PCR was done using the BIORAD iCycler. The CellQuanti-MTT^{TM} cell viability assay kit and the QuantiChrom^{TM} alkaline phosphatase (ALP) assay kit were purchased from BioAssay Systems (US).

2.2 Preparation of substrates

Ti substrates were prepared as previously described [8]. Briefly, Ti foils of 0.52-mm thickness were cut to a size of 1 cm × 1 cm, and cleaned with sandpaper, followed by sonication sequentially in Knoll's reagent (4.0% HF, 7.2% HNO_3, 88.8% water), dichloromethane, acetone and water. After surface passivation in 40% HNO_3, dopamine was anchored to the surface of the Ti substrates as described elsewhere [9]. Subsequently, the substrates were immersed in a stirred 3% glutaraldehyde solution at room temperature overnight. Glutaraldehyde serves as a cross-linker, providing the reactive aldehyde groups for covalently bonding dopamine and CS. Unbound glutaraldehyde was removed by rinsing the substrates in water. CS was dissolved in a 0.1% dilute acetic acid to a concentration of 5 mg/ml. The glutaraldehyde-treated substrates were then immersed in the CS solution. Imine bonds are formed between the aldehyde groups on the Ti surface and the primary amino groups at the C-2 position of CS. The resulting substrates were then washed with water and dried under vacuum. Some of the chitosan-grafted Ti substrates were then individually coated with BMP2 at a concentration of 50 μg/ml and allowed to air dry in a sterile environment. Following which, the substrates were rinsed 3 times with sterile PBS to remove unattached BMP2 and left to air dry in a sterile environment before use. We used a BMP2 concentration of 50 μg/ml to coat the titanium substrates to ensure that the growth factor was adequately adsorbed onto the substrate surface. The substrates are denoted Ti (pristine titanium), Ti-CS (chitosan-grafted Ti) and Ti-CS-BMP2 (BMP2-coated, chitosan-grafted Ti) in subsequent discussions. All the biological assays were done in duplicates, and each assay was done twice.

2.3 Characterization

The chemical composition of the surfaces was analyzed by X-ray photoelectron spectroscopy (XPS) on an AXIS HSi spectrometer (Kratos Analytical Ltd., UK) with an Al Kα X-ray source (1486.6 eV photons). The details for the XPS measurements are similar to those reported earlier [10]. All binding energies (BEs) were referenced to the C 1s hydrocarbon peak at 284.6 eV. In the peak synthesis, the line width (full width at half-maximum) of the Gaussian peaks was kept constant for all components in a particular spectrum.

2.4 Stability and surface density of adsorbed BMP2 on Ti-CS-BMP2 substrates

To assess the stability of BMP2 on the Ti-CS-BMP2 substrates, the substrates were placed individually in a 24-well plate containing 1 ml of PBS per well. The intensity of the BMP2 on the substrate surface was assessed on days 0, 1, 3, 5 and 7. At each respective point in time, the substrates were removed and placed in a new well each. One millilitre of PBS was then added to each well, and the adsorbed BMP2 was gently scrapped off the substrate surface with 5 strokes of a cell scraper. The amount of BMP2 in solution was then measured using the Bio-Rad DC Protein Assay, following the manufacturer's instructions. The colour intensity of the reaction mixture was measured at 700 nm using an absorbance plate reader after an incubation period of 15 min. The amount of attached BMP2 on day 0 is the initial coating density, while those on days 1, 3, 5 and 7 give an indication of the stability of BMP2 on the Ti-CS-BMP2 substrate surface. The absolute amount of BMP2 adsorbed onto the substrate surface, that is, the initial coating density, was measured as described above, using a

series of BMP2 solutions of known concentrations as standards.

2.5 Cell culture

Bone marrow was taken with written consent from patients undergoing surgery for rheumatoid arthritis. The marrow was washed 3 times with copious Hanks Balanced Salt Solution (HBSS). Erythrocytes were lysed with a 0.8% ammonium chloride solution and the bone marrow cells were centrifuged at 300*g* for 10 min. The cell pellet was then washed 3 times with HBSS, resuspended and cultured in a medium comprising Minimal Essential Medium-alpha (MEM-α), supplemented with 10% foetal bovine serum (FBS), L-glutamine (2 mmol/l), penicillin (100 U/ml) and streptomycin sulphate (100 μg/ml), and incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Unattached cells were removed on the following day. The attached cells were then denoted BMMSCs. Cell colonies that formed during culture were identified and removed by gentle trypsinization (0.25% trypsin-EDTA). Trypsin was removed by centrifugation. Each colony of BMMSCs was re-seeded in a new culture flask. Half of the culture medium was replaced by a fresh aliquot of medium once every 2 days. The BMMSCs were then allowed to grow to about 75% confluence before use. Passage 2 cells were used throughout the study. Attached cells were detached by trypsinization and resuspended in fresh culture medium for subsequent experiments described below.

Bone chip-derived osteoblasts (BC-OB) were obtained from bone chips of the same patients. The bone chips were washed 3 times in HBSS, cut into smaller fragments before being placed in tissue culture flasks and cultured under the same conditions as BMMSCs. Upon reaching approximately 75% confluence, the bone chips were removed and the cells were washed 3 times with HBSS, trypsinized and reseeded in new tissue culture flasks for further expansion. Passage 2 BC-OBs were used for RT-PCR.

Although the experiments were done using bone marrow donated by three different patients, the results are a representative from one sample which had consistent cell morphology and a high cell proliferation rate by passage 2.

2.6 Cell attachment

Cell attachment on the various titanium substrates was evaluated by counting the number of attached cells 6 h after cell seeding. The substrates were placed into a 24-well plate and seeded with BMMSCs at a density of 5,000 cells/cm^2. The number of attached cells on Ti, Ti-CS and Ti-CS-BMP2 was evaluated, and compared to the number of attached cells on the bottom of a similar well without any titanium substrate (control). At the time of cell counting, unattached cells were rinsed off with PBS. The attached cells were detached and counted using a haemocytometer.

2.7 Cell proliferation

Cell proliferation on the substrates was evaluated by counting the number of attached cells on days 1, 3, 5 and 7. The number of attached cells on the bottom of a similar well without any titanium substrate was counted at the respective points in time and was used as a control. At each designated point in time, the unattached cells were rinsed off with PBS. Attached cells were detached and counted using a haemocytometer. The number of attached cells is reported as number of cells/cm^2.

2.8 Flow cytometry

Cells were detached by gentle trypsinization and then incubated in PBS with 2% FBS for 10 min in an ice bath. One microlitre each of CD44-FITC, CD105-PE, CD271-FITC, CD34-FITC and CD45-FITC antibodies was added to the cells suspended in 100 μl of PBS with 2% FBS each in separate tubes. The tubes were incubated for 10 min in an ice bath in the dark. The cells were then washed and resuspended in 1 ml of PBS, and analysed using flow cytometry. A total of 10,000 events were collected for the analysis of each marker (Table 1).

2.9 Cytotoxicity assay

The MTT assay was carried out at day 7 of culture of the cells on the different titanium substrates to assess the effect of the substrates on the viability of the BMMSCs. The assay is based on the conversion of yellow MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to purple formazan in the mitochondria of living cells. The insoluble purple formazan product was dissolved in a solubilization reagent supplied in the assay kit. The absorbance of the resulting coloured solution was measured at 570 nm on an absorbance plate reader.

Table 1 This analysis shows the profile of the surface markers of the bone marrow stromal cells

Marker	CD34	CD45	CD271	CD44	CD105
Percentage	1.3	1.1	3.4	64.0	86.0

Flow cytometry analysis of these passage 2 BMMSCs shows the presence of mesenchymal stem cell markers CD271, CD44 and CD105, suggesting that there is a population of progenitors cells in bone marrow that can possibly contribute to osteoblast differentiation and osteointegrate with the titanium substrates. A small fraction of CD34+ and CD45+ haematopoietic cells were present among the BMMSCs

2.10 Alkaline phosphatase assay

The level of alkaline phosphatase (ALP) of the cells was assessed using a ALP assay kit. The ALP assay is used in this study to assess the level of osteoblast differentiation from BMMSCs. This assay relies on the ALP hydrolysis of *p*-nitrophenyl phosphate into a yellow product, the intensity of which was measured at 405 nm on an absorbance plate reader. Briefly, BMMSCs were seeded onto the substrates at a density of 5,000 cells/cm^2. At day 7 of culture, the cell layers were washed with HBSS and gently scraped off from the surfaces of the substrates. HBSS was removed by centrifugation and cell lysis buffer was then added to the cells. The cells were sonicated to disrupt the cell membranes. After sonication, cellular debris was removed by centrifugation and aliquots of the cell lysates were collected for the analysis of the ALP activity and the quantification of total protein level. The assay was carried out at room temperature. The colour intensity of the reaction mixture was measured at 4-min intervals. The ALP activity of each sample was calculated according to a formula provided in the kit. The readings at the 20-min interval were used to determine the respective levels of ALP activity. ALP activity was determined as the rate of *p*-nitrophenol liberation from *p*-nitrophenyl phosphate, normalized with respect to the total protein content obtained from the same cell lysate, and expressed as number of IU of *p*-nitrophenol formation per minute per milligram of total proteins (IU min^{-1} mg^{-1} protein).

2.11 Reverse transcription-polymerase chain reaction

RNA was extracted from cells on day 10 of culture using the NucleoSpin® RNA/Protein isolation kit (Macherey-Nagel, GmbH, Germany) according to the manufacturer's instructions. Reverse transcription of mRNA to cDNA and subsequent cDNA amplification was done using the TITANIUM™ One-step RT-PCR Kit (Clontech Laboratories, Inc., US), through one cycle of 50°C for 1 h and 94°C for 5 min. This was followed by 30 cycles of 94°C for 30 s, 65°C for 30 s and 68°C for 1 min, with a final extension at 68°C for 2 min. The expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used to check RNA integrity. The amplified products were subjected to electrophoresis in a 2% agarose gel and stained with ethidium bromide (Sigma–Aldrich, Singapore). Cells grown on Ti-CS-BMP2 substrates were compared with BC-OBs and untreated BMMSCs, both cultured in normal tissue culture flasks. Densitometry was done to quantify the relative levels of the bone-related genes, normalized to the respective levels of GADPH. Primers used for the cDNA amplification and the annealing temperatures are listed in Table 2.

2.12 Alizarin red staining

Cells were grown on the titanium substrates for 21 days and removed by trypsinization, then re-seeded into individual wells of a new 24-well plate. Forty-eight hours after re-plating, the cells were stained with 1% alizarin red S for 2 min and then washed with PBS. The stained cells were observed under a light microscope.

2.13 Statistical analysis

The results were assessed statistically using one-way analysis of variance. Statistical significance was accepted at $P < 0.05$.

3 Results

3.1 Stability and surface density of adsorbed BMP2 on Ti-CS-BMP2 substrates

Others have physically modified the surface of the titanium substrate and allowed BMP2 to be adsorbed onto the surface. Upon immersion of the substrate in a solution, only a small percentage of the initially adsorbed amount of BMP2

Table 2 Primers used for the reverse transcription-polymerase chain reaction of bone-related genes

Gene (Ascension number)	Sequence	T_{anneal} (°C)	Product size (bp)
RUNX2 (NM_004348)	Forward tttgcactgggtcatgtgtt	58	156
	Reverse tggctgcattgaaaagactg	58	
Collagen I (NM_000088)	Forward ccaaatctgtctccccagaa	60	214
	Reverse tcaaaaacgaaggggagatg	58	
BMP2 (NM_001200)	Forward cccagcgtgaaaagagagac	62	168
	Reverse gagaccgcagtccgtctaag	64	
Osteocalcin (NM_199173)	Forward gtgcagagtccagcaaaggt	62	175
	Reverse tcagccaactcgtcacagtc	62	
GAPDH (NM_002046)	Forward gagtcaacggatttggtcgt	60	238
	Reverse ttgattttggagggatctcg	60	

remained firmly attached to the substrate surface [5]. We have covalently linked chitosan onto the surface of titanium using a linker, as previously reported [4]. We then immersed these substrates in a solution containing a high concentration of BMP2 to allow sufficient BMP2 to be adsorbed onto the substrate surface. BMP2 adsorption on the Ti-CS substrates is probably via strong electrostatic interaction. We expected that most of the BMP2 molecules would be detached from the substrate surface upon immersion in a liquid. We found that only about 1%, or 500 ng of the initial coating solution concentration of 50 μg, was firmly attached onto the substrate surface (results not shown). Approximately 81% of the BMP2 that was initially attached on day 0 remained attached to the substrate surface on day 1. The attached BMP2 was slowly released into solution over time, at a rate of about 2%/day from day 3 to day 7, as is shown by the results of the surface density assessment in Fig. 1. From Fig. 1, it can also be seen that the BMP2 rate of release was not consistent from day 0 to the start of day 3. We speculate that this could be due to the overcrowding of BMP2 molecules on the substrate surface, resulting in some of the BMP2 molecules being loosely attached. By day 7, the amount of attached BMP2 was still more than 50% of the initial coating density, suggesting that enough BMP2 can remain attached on the Ti-CS surface for a length of time, and released slowly to support osteoblast differentiation.

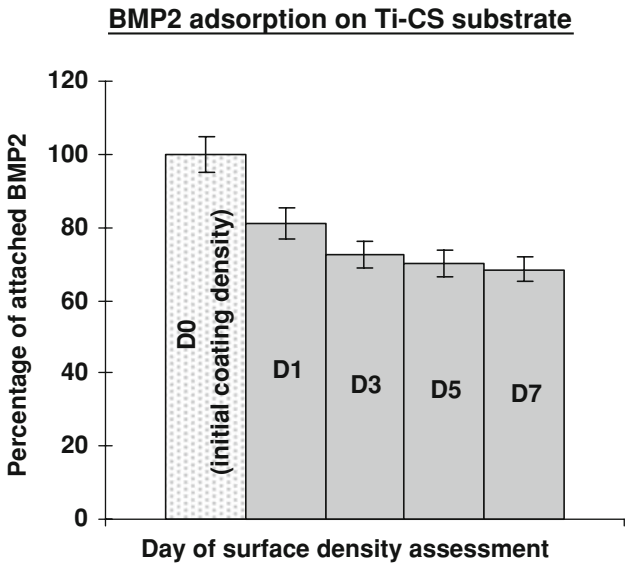

Fig. 1 Data reported as percentage of BMP2 that remained adsorbed on the Ti-CS substrate ($n = 2$). Error bars represents the standard deviations of each group. About 70% of the initial coating of BMP2 remained attached to the Ti-CS substrate surface after 7 days of submersion in PBS, indicating that BMP2 can potentially remain attached for a sufficient period of time to enhance osteoblast differentiation

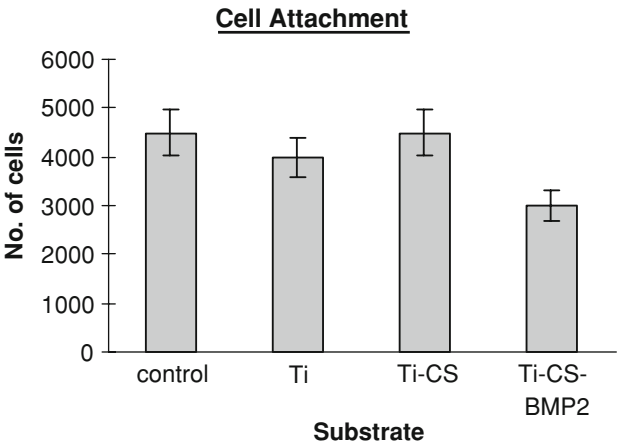

Fig. 2 The number of attached BMMSCs on each type of titanium substrate ($n = 2$) was evaluated on day 1. Error bars represent the standard deviations of each group. As shown, BMMSCs can adhere to the titanium substrates as effectively as on the plastic surface of culture flasks, suggesting that bone implants constructed with these substrates can possibly support BMMSC adhesion on the prosthesis, where further cellular developments can occur

3.2 Cell attachment and proliferation

The number of BMMSCs attached on the various titanium surfaces was assessed 6 h after cell seeding, and compared to the control, where the cells directly attached to the bottom of the culture well (Fig. 2). Cell attachment on Ti-CS substrates was comparable to that in the control (without any titanium substrate), while the number of cells attached to the pristine Ti substrate was less compared to that on either the Ti-CS substrate or in the control. This suggests that the chitosan that is grafted onto the titanium enhances initial cell attachment. However, the Ti-CS-BMP2 substrates had the least number of attached cells. This can probably be attributed to the fact that the adsorbed BMP2 molecules occupied a proportion of the available area on the chitosan-grafted titanium surface, altering its surface topology. Substrate surface topology is known to affect cell attachment [11]. Cell proliferation progressed steadily over the following 7 days of culture, as shown in Fig. 4. Except for the control on day 7, the cell proliferation rates were not significantly different on all the titanium substrates and in the controls ($P < 0.05$). This apparent anomaly could be due to the fact that cells on the substrates were undergoing differentiation, as differentiating cells are known to slow down in proliferation rate or even leave the cell cycle [12]. Others have shown that cell attachment is significantly affected by the presence of phosphate and apatite groups on titanium surface [13]. This suggests that the presence of certain types of molecules on the titanium substrate surface can affect the degree of cell attachment. Besides the presence of certain preferred molecules, the technique employed in applying coatings on a titanium

substrate can alter the substrate surface topology, and hence, cell attachment efficiency [11]. In comparison, the chitosan on our Ti-CS substrate is a stable, covalently attached layer which should confer anti-bacterial properties to the titanium substrate for an extended period of time, while promoting cell attachment on the substrate surface.

3.3 Flow cytometry

Passage 2 BMMSCs were subjected to flow cytometry analysis after being separately stained with CD271, CD44, CD105, CD34 and CD45 antibodies to assess their lineage. About 64% of the cells were CD44+ while approximately 86% of them were CD105+. Abount 1.3% of CD34+ cells and 1.1% of CD45+ cells were present among the BMMSCs. The rare population of CD271 cells which has been described as one of the most specific markers of BMMSCs constituted about 3.4% of the cell population [14, 15].

3.4 Cytotoxicity

Cell viability on the 3 types of substrates was assessed using the MTT assay (Fig. 3), to give an indication of the effect of the substrates on the survival and proliferation of the attached cells. The assay was done on day 7 of culture and the results suggest that all the 3 types of titanium substrates do not seem to be cytotoxic to the cells, and can support cell growth and proliferation almost as effectively as plastic culture flasks (control), as shown in Fig. 4.

3.5 Alkaline phosphatase activity

The results in Fig. 5 indicate that osteoblast differentiation occurred on all the substrates, with the ALP activity of the

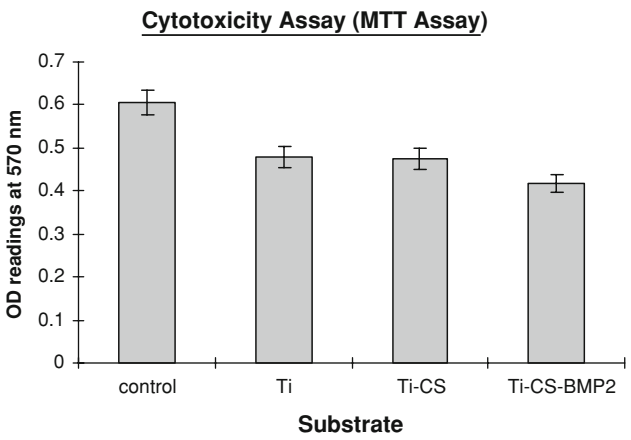

Fig. 3 The MTT assay shows that the three types of titanium substrates ($n = 2$) do not adversely affect cell viability, suggesting that these substrates are probably not cytotoxic in vivo, and can possibly be used to fabricate bone implants. Error bars represent the standard deviations of each group

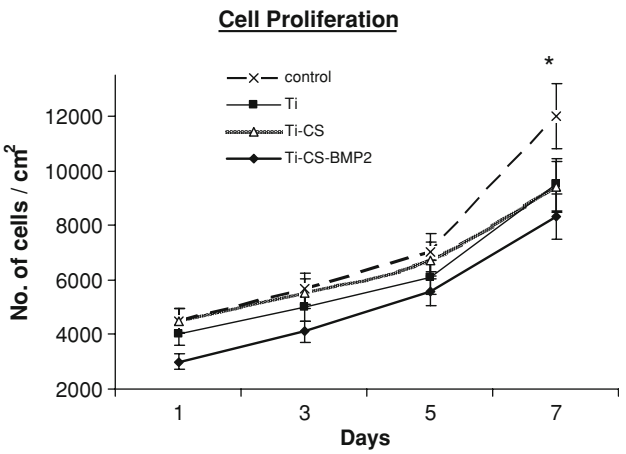

Fig. 4 The ability of the titanium substrates to support cell proliferation was assessed by counting the number of attached BMMSCs on each type of substrate ($n = 2$) on days 1, 3, 5 and 7. The cell proliferation rates are consistent on all the three types of substrates. By day 7, most of the cells on the substrates are probably differentiating, hence the slow-down in proliferation rates as compared with that in the control (denoted by *). Error bars represent the standard deviations of each group

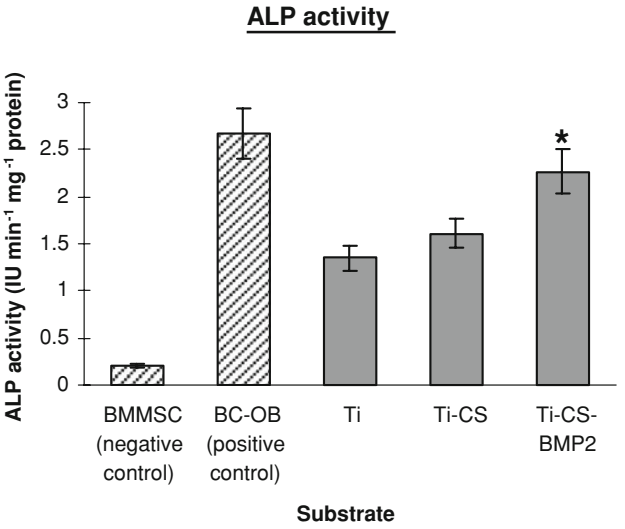

Fig. 5 ALP activity in the cells attached to each type of titanium substrate ($n = 2$) indicates osteoblast differentiation. Although cells on the Ti-CS substrates had a higher ALP activity than those on the Ti substrates, this was not significant. Only cells on the Ti-CS-BMP2 substrate show a significantly higher ALP activity than cells on either of the other 2 types of substrates (denoted by *), indicating that CS and BMP2 may synergistically work to enhance BMMSC differentiation into osteoblasts. In contrast, BMMSCs cultured in normal tissue culture flasks showed almost no ALP activity. The ALP activity of BC-OBs serves as a positive control. Error bars represent the standard deviations of each group

cells on the various substrates in the ascending order Ti, Ti-CS, and Ti-CS-BMP2. Chitosan has been shown to enhance the ALP activity of osteoblasts [16, 17]. Our

results show that although there was a higher ALP activity in the Ti-CS cells compared with the Ti cells, the increase was not significant. However, Ti-CS-BMP2 cells showed a significantly higher ALP activity over both Ti cells and Ti-CS cells ($P < 0.05$). Thus, the results suggest that CS and BMP2 on the Ti-CS-BMP2 substrate surface may have a synergistic effect in enhancing osteoblast differentiation from BMMSCs. Based on this result, RT-PCR was carried out using BMMSCs cultured on the Ti-CS-BMP2 substrate.

3.6 RT-PCR

RT-PCR was carried out to assess the expression of the genetic markers, Runx2, collagen type I (Col I) and osteocalcin (OCN), to confirm osteoblast differentiation on the Ti-CS-BMP2 substrate (Fig. 6a). The results were compared with those from BC-OBs and untreated BMMSCs cultured in normal tissue culture flasks. In addition, BC-OBs were also assessed for the expression of endogenous BMP2, a key growth factor involved in bone formation, to confirm the bone cell lineage of the BC-OBs. Figure 6b shows the relative levels of expression of the 3 genes in BC-OBs and Ti-CS-BMP2 cells, normalized to the expression level of GAPDH.

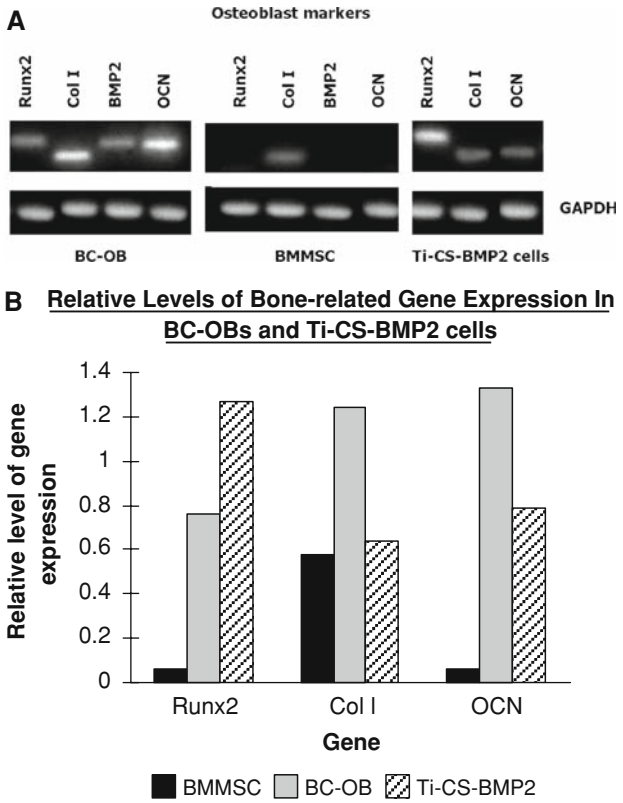

Fig. 6 (**a** and **b**) RT-PCR of Runx2, Col I and OCN suggests that Ti-CS-BMP2 cells are undergoing osteoblast differentiation, unlike BC-OBs (positive control), which are mature osteoblasts. This is evident from the comparatively higher level of Runx2, indicating a higher transcription activity level of this gene, and the relatively lower levels of Col I and OCN, indicating that the Ti-CS-BMP2 cells are not fully differentiated osteoblasts yet. Conversely, untreated, BMMSCs cultured in plastic tissue culture flasks (negative control) do not differentiate and hence have no detectable level of bone-related markers except for Col I

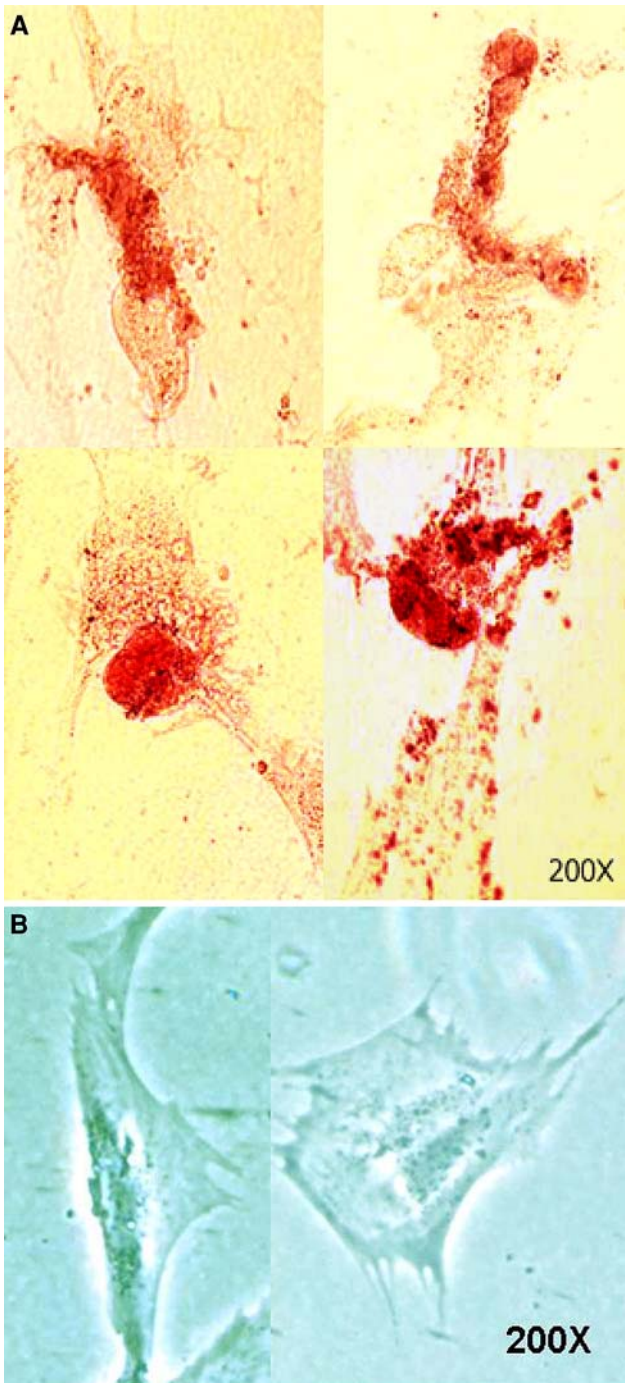

Fig. 7 Ti-CS-BMP2 cells that stain positive for the presence of calcium deposits with alizarin red S indicate osteoblast differentiation (**a**), while untreated, cultured BMMSCs do not differentiate and hence stain negative for alizarin red S (**b**)

3.7 Alizarin red staining

The Ti-CS-BMP2 cells were stained with Alizarin red S to detect calcium deposits in the cells. Figure 7a shows the some of the positively stained cells. Untreated BMMSCs stained negative with Alizarin red S, as shown in Fig. 7b.

4 Discussion

MSCs have been reported to retain their undifferentiated phenotype and remain capable of osteogenic differentiation during ex vivo expansion through multiple passages [18]. Their surface marker expression profiles remain consistent, and their gene expression profiles do not change significantly during long-term expansion [18]. The well-established MSC markers, CD44 and CD105, used to confirm the MSC lineage of the BMMSCs in this study reveal that there is a population of relatively undifferentiated progenitors that can possibly contribute to osteoblast differentiation [18–20]. In addition, we found a small population of the cells that were also CD271+. CD271 is one of the most specific and novel markers of BMMSCs [14]. CD271+ cells are highly proliferative, and possess multilineage differentiation potential [15, 20, 21]. Unfortunately, CD271+ cells are rare in a population of bone marrow-derived cells, and would have to be immunomagnetically isolated, and then expanded in vitro. Thus, a relatively large volume of bone marrow would have to be extracted in order that a feasible number of CD271+ cells can be magnetically isolated to constitute the starting cells for in vitro expansion.

While osteoblast differentiation from BMMSCs may occur on the surface of titanium substrates, it is paramount that BMMSCs must first be able to firmly attach to the substrate surface. This is to ensure that osteointegration of a bone implant can take place in vivo. MSCs are known to adhere strongly to plastic surfaces and this characteristic has enabled them to be easily isolated and investigated for potential therapeutic applications [22]. Our patient-derived BMMSCs also similarly adhere to plastic surfaces and to the various titanium substrates. Firm cell attachment is evidenced by the prolonged trypsinization required to detach them from the substrates. Together with the consistent cell proliferation rates, this indicates that BMMSCs can be a potential source of progenitors to initiate osteointegration with functionalized titanium-based implants in vivo. However, such surface-modified implants must be biocompatible. The results of the MTT assay show that our titanium-based substrates do not adversely affect cell viability, indicating that these substrates probably would not be cytotoxic in vivo.

An assessment of the ALP activity of the cells on the various types of substrate surfaces on day 7 of culture suggests that osteoblast differentiation can possibly occur soon after cell attachment. While others have used calcium phosphate-coated, collagen-coated, or chondroitin sulphate-coated titanium to enhance the retention of BMP2 on the substrate surface, we allowed BMP2 to be adsorbed onto our chitosan-grafted titanium substrates [6, 23, 24]. Besides being able to promote osteoblast differentiation, chitosan also possesses anti-bacterial properties [25–28]. Thus, we hypothesized that chitosan and BMP2 would act synergistically to improve osteoblast differentiation on titanium surfaces. Based on the results of the ALP assay, we believe that our Ti-CS-BMP2 substrates would be the most osteogenic among our 3 types of titanium substrates. We proceeded to assess the expression levels of three bone genetic markers in the BMMSCs grown on Ti-CS-BMP2 substrates. RT-PCR was carried out to assess the expression levels of three key bone markers, Runx2, Col I and OCN. Runx2 is the transcription gene of the osteoblast specific protein, osteocalcin. Together, Runx2 and OCN are the key regulators of osteoblast differentiation and function [29–31]. Runx2 is regulated by a number of factors including bone morphogenetic protein (BMP). It functions as a scaffold protein for nucleic acids and regulatory factors involved in skeletal gene expression. Osteocalcin is the major noncollagenous protein of bone matrix [32]. Interestingly, besides being used as a biomarker of osteoblast differentiation, this gene has been implicated in human osteoarthritis and its presence could possibly mark the stage of the development of this disease at a point of dysregulation of cellular behaviour and failure of repair mechanisms [32]. Thus, the detection of OCN in the serum may potentially be used as a clinical indicator of the need for a bone implant. Collagen I is the major constituent in bone extracellular matrix [33]. Cells of the bone lineage express Col I in all their developmental stages. Collagen I interacts with the $\alpha_2\beta_1$ integrin receptor on the cell membrane to mediate transduction of extracellular signals into the cell [34]. The interruption of this collagen–integrin interaction inhibits osteoblast differentiation from bone marrow cells [34]. The RT-PCR results show that these three key genes were expressed in the Ti-CS-BMP2 cells, and only Col I was expressed in the untreated BMMSCs, indicating that the Ti-CS-BMP2 substrate promotes osteoblast differentiation in the BMMSCs that adhered to its surface. A densitometry of the band intensities of Runx2, Col I and OCN suggests that the Ti-CS-BMP2 cells were in the early stages of osteoblast differentiation (Fig. 6b). This is evident from the relatively lower levels of Col I and OCN in the Ti-CS-BMP2 cells as compared with those from the BC-OBs. Furthermore, the relatively higher level of the transcription factor, Runx2, in

the Ti-CS-BMP2 cells suggests a higher gene transcription activity level of bone-related genes than that in the BC-OBs. Additionally, the BC-OBs were assessed for the expression of endogenous BMP2, which is a key gene in bone cells, as BMP2 also serves to regulate the Runx2 gene. Finally, the Ti-CS-BMP2 cells were stained with alizarin red S to detect calcium deposits in the cells.

Besides chemically modifying the substrate surface, others have also physically modified the titanium substrate surface to enhance the adsorption of BMP2 [5]. While most of these modified surfaces have been reported to support osteoblast differentiation, others have also reported that immobilized BMP2 on titanium substrates with biomimetic coatings does not enhance bone formation [6]. Our method of immobilizing BMP2 onto chitosan-grafted titanium surfaces is convenient and effective in promoting BMMSC-derived osteoblast differentiation. Although only a small proportion of the initial amount of BMP2 used for adsorption onto the chitosan-grafted titanium surface were firmly attached, this amount was sufficient to promote osteoblast differentiation. Nevertheless, one very distinct advantage of our chitosan-grafted titanium substrates is their bacteria-resistant property. Our Ti-CS-BMP2 substrate therefore has the potential to be used to fabricate anti-bacteria, osteoconductive bone implants.

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References

1. C.M. Kolf, E. Cho, R.S. Tuan, Mesenchymal stromal cells. Biology of adult mesenchymal stem cells: regulation of niche, self-renewal and differentiation. Arthritis Res. Ther. **9**, 204–213 (2007). doi:10.1186/ar2116
2. S. Ohnishi, H. Ohgushi, S. Kitamura, N. Nagaya, Mesenchymal stem cells for the treatment of heart failure. Int. J. Hematol. **86**, 17–21 (2007). doi:10.1532/IJH97.07041
3. E.I. Rabea, M.E. Badawy, C.V. Stevens, G. Smagghe, W. Steurbaut, Chitosan as antimicrobial agent: applications and mode of action. Biomacromolecules **4**, 1457–1465 (2003). doi:10.1021/bm034130m
4. Z. Shi, K.G. Neoh, E.T. Kang, C. Poh, W. Wang, Bacterial adhesion and osteoblast function on titanium with surface-grafted chitosan and immobilized RGD peptide. J. Biomed. Mater. Res. (2007)
5. J. Hall, R.G. Sorensen, J.M. Wozney, U.M. Wikesjo, Bone formation at rhBMP-2-coated titanium implants in the rat ectopic model. J. Clin. Periodontol. **34**, 444–451 (2007). doi:10.1111/j.1600-051X.2007.01064.x
6. H. Schliephake, A. Aref, D. Scharnweber, S. Bierbaum, S. Roessler, A. Sewing, Effect of immobilized bone morphogenic protein 2 coating of titanium implants on peri-implant bone formation. Clin. Oral Implants Res. **16**, 563–569 (2005). doi:10.1111/j.1600-0501.2005.01143.x
7. H. Zhang, M. Oh, C. Allen, E. Kumacheva, Monodisperse chitosan nanoparticles for mucosal drug delivery. Biomacromolecules **5**, 2461–2468 (2004). doi:10.1021/bm0496211
8. T.A. Barber, S.L. Golledge, D.G. Castner, K.E. Healy, Peptide-modified p(AAm-co-EG/AAc) IPNs grafted to bulk titanium modulate osteoblast behavior in vitro. J. Biomed. Mater. Res. **64**, 38–47 (2003). doi:10.1002/jbm.a.10321
9. X. Fan, L. Lin, J.L. Dalsin, P.B. Messersmith, Biomimetic anchor for surface-initiated polymerization from metal substrates. J. Am. Chem. Soc. **127**, 15843–15847 (2005). doi:10.1021/ja0532638
10. Z. Shi, K.G. Neoh, E.T. Kang, Antibacterial activity of polymeric substrate with surface grafted viologen moieties. Biomaterials **26**, 501–508 (2005). doi:10.1016/j.biomaterials.2004.02.060
11. D.S. Kommireddy, S.M. Sriram, Y.M. Lvov, D.K. Mills, Stem cell attachment to layer-by-layer assembled TiO_2 nanoparticle thin films. Biomaterials **27**, 4296–4303 (2006). doi:10.1016/j.biomaterials.2006.03.042
12. G. Plickert, M. Kroiher, A. Munck, Cell proliferation and early differentiation during embryonic development and metamorphosis of Hydractinia echinata. Development **103**, 795–803 (1988)
13. Y. Yang, J.D. Bumgardner, R. Cavin, D.L. Carnes, J.L. Ong, Osteoblast precursor cell attachment on heat-treated calcium phosphate coatings. J. Dent. Res. **82**, 449–453 (2003)
14. H.J. Buhring, V.L. Battula, S. Treml, B. Schewe, L. Kanz, W. Vogel, Novel markers for the prospective isolation of human MSC. Ann. N. Y. Acad. Sci. **1106**, 262–271 (2007). doi:10.1196/annals.1392.000
15. N. Quirici, D. Soligo, P. Bossolasco, F. Servida, C. Lumini, G.L. Deliliers, Isolation of bone marrow mesenchymal stem cells by anti-nerve growth factor receptor antibodies. Exp. Hematol. **30**, 783–791 (2002). doi:10.1016/S0301-472X(02)00812-3
16. T. Matsunaga, K. Yanagiguchi, S. Yamada, N. Ohara, T. Ikeda, Y. Hayashi, Chitosan monomer promotes tissue regeneration on dental pulp wounds. J. Biomed. Mater. Res. **76**, 711–720 (2006). doi:10.1002/jbm.a.30588
17. S. Yamada, N. Ohara, Y. Hayashi, Mineralization of matrix vesicles isolated from a human osteosarcoma cell line in culture with water-soluble chitosan-containing medium. J. Biomed. Mater. Res. **66**, 500–506 (2003). doi:10.1002/jbm.a.10009
18. B. Kulterer, G. Friedl, A. Jandrositz et al., Gene expression profiling of human mesenchymal stem cells derived from bone marrow during expansion and osteoblast differentiation. BMC Genomics **8**, 70–84 (2007). doi:10.1186/1471-2164-8-70
19. D. Campoccia, L. Montanaro, C.R. Arciola, The significance of infection related to orthopedic devices and issues of antibiotic resistance. Biomaterials **27**, 2331–2339 (2006). doi:10.1016/j.biomaterials.2005.11.044
20. E.A. Jones, S.E. Kinsey, A. English et al., Isolation and characterization of bone marrow multipotential mesenchymal progenitor cells. Arthritis Rheum. **46**, 3349–3360 (2002). doi:10.1002/art.10696
21. E.A. Jones, A. English, K. Henshaw et al., Enumeration and phenotypic characterization of synovial fluid multipotential mesenchymal progenitor cells in inflammatory and degenerative arthritis. Arthritis Rheum. **50**, 817–827 (2004). doi:10.1002/art.20203
22. M. Kassem, B.M. Abdallah, Human bone-marrow-derived mesenchymal stem cells: biological characteristics and potential role in therapy of degenerative diseases. Cell Tissue Res. **331**, 157–163 (2007)
23. Y. Liu, K. de Groot, E.B. Hunziker, BMP-2 liberated from biomimetic implant coatings induces and sustains direct ossification in an ectopic rat model. Bone **36**, 745–757 (2005). doi:10.1016/j.bone.2005.02.005
24. Y. Liu, E.B. Hunziker, P. Layrolle, J.D. De Bruijn, K. De Groot, Bone morphogenetic protein 2 incorporated into biomimetic coatings retains its biological activity. Tissue Eng. **10**, 101–108 (2004). doi:10.1089/107632704322791745

25. J. Fu, J. Ji, W. Yuan, J. Shen, Construction of anti-adhesive and antibacterial multilayer films via layer-by-layer assembly of heparin and chitosan. Biomaterials **26**, 6684–6692 (2005). doi:10.1016/j.biomaterials.2005.04.034
26. M.W. Huh, I. Kang, D.H. Lee, W.S. Kim, D.H. Lee, L.S. Park, K.E. Min, K.H. Seo, Surface characterization and antibacterial activity of chitosan-grafted poly(ethylene terephthalate) prepared by plasma glow discharge. J. Appl. Polym. Sci. **81**, 2769–2778 (2001). doi:10.1002/app.1723
27. H.K. No, N.Y. Park, S.H. Lee, S.P. Meyers, Antibacterial activity of chitosans and chitosan oligomers with different molecular weights. Int. J. Food Microbiol. **74**, 65–72 (2002). doi:10.1016/S0168-1605(01)00717-6
28. Z. Shi, K.G. Neoh, E.T. Kang, W. Wang, Antibacterial and mechanical properties of bone cement impregnated with chitosan nanoparticles. Biomaterials **27**, 2440–2449 (2006). doi:10.1016/j.biomaterials.2005.11.036
29. P. Ducy, Cbfa1: a molecular switch in osteoblast biology. Dev. Dyn. **219**, 461–471 (2000). doi:10.1002/1097-0177(2000)9999:9999<::AID-DVDY1074>3.0.CO;2-C
30. T. Komori, Cbfa1/Runx2, an essential transcription factor for the regulation of osteoblast differentiation. Nippon Rinsho. **60**(Suppl 3), 91–97 (2002)
31. T.M. Schroeder, E.D. Jensen, J.J. Westendorf, Runx2: a master organizer of gene transcription in developing and maturing osteoblasts. Birth Defects Res. C: Embryo Today **75**, 213–225 (2005). doi:10.1002/bdrc.20043
32. O. Pullig, G. Weseloh, D. Ronneberger, S. Kakonen, B. Swoboda, Chondrocyte differentiation in human osteoarthritis: expression of osteocalcin in normal and osteoarthritic cartilage and bone. Calcif. Tissue Int. **67**, 230–240 (2000). doi:10.1007/s002230001108
33. S. Gronthos, A.C. Zannettino, S.E. Graves, S. Ohta, S.J. Hay, P.J. Simmons, Differential cell surface expression of the STRO-1 and alkaline phosphatase antigens on discrete developmental stages in primary cultures of human bone cells. J. Bone Miner. Res. **14**, 47–56 (1999). doi:10.1359/jbmr.1999.14.1.47
34. M. Mizuno, Y. Kuboki, Osteoblast-related gene expression of bone marrow cells during the osteoblastic differentiation induced by type I collagen. J. Biochem. **129**, 133–138 (2001)