Title: Topography of calcium phosphate ceramics regulates primary cilia length and TGF receptor recruitment associated with osteogenesis

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Keywords: calcium phosphate ceramic; topography; mesenchymal stromal cell; primary cilia; TGFβ; bone.

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Abstract: The surface topography of synthetic biomaterials is known to play a role in material-driven osteogenesis. Recent studies show that TGFβ signaling also initiates osteogenic differentiation. TGFβ signaling requires the recruitment of TGFβ receptors (TGFβR) to the primary cilia. In this study, we hypothesize that the surface topography of calcium phosphate ceramics regulates stem cell morphology, primary cilia structure and TGFβR recruitment to the cilium associated with osteogenic differentiation. We developed a 2D system using two types of tricalcium phosphate (TCP) ceramic discs with identical chemistry. One sample had a surface topography at micron-scale (TCP-B, with a bigger surface structure dimension) whilst the other had a surface topography at submicron scale (TCP-S, with a smaller surface structure dimension). In the absence of osteogenic differentiation factors, human bone marrow stromal cells (hBMSCs) were more spread on TCP-S than on TCP-B with alterations in actin organization and increased primary cilia prevalence and length. The cilia elongation on TCP-S was similar to that observed on glass in the presence of osteogenic media and was followed by recruitment of transforming growth factor-β RI (p-TGFβ RI) to the cilia axoneme. This was associated with enhanced osteogenic differentiation of hBMSCs on TCP-S, as shown by alkaline phosphatase staining and gene expression for key osteogenic markers in the absence of additional osteogenic growth factors. Similarly, in vivo after a 12-week intramuscular implantation in dogs, TCP-S induced bone formation while TCP-B did not. It is most likely that the surface topography of calcium phosphate ceramics regulates primary cilia length and ciliary recruitment of p-TGFβ RI associated with osteogenesis and bone formation. This bioengineering control of osteogenesis via primary cilia modulation may represent a new type of biomaterial-based ciliotherapy for orthopedic, dental and maxillofacial surgery applications.
Response letter

Acta Biomaterialia- Decision on Manuscript ID AB-16-2689
Title: Topography of calcium phosphate ceramics regulates primary cilia length and TGF receptor recruitment associated with osteogenesis

Dear Editor,

We appreciate your time and effort for reviewing our manuscript. We would also like to express our gratitude to the reviewers for their invaluable and constructive comments to help us significantly improve our manuscript. We have now fully addressed their comments in the response letter and revised the manuscript accordingly. For convenience, the reviewers’ comments are shown in blue italic in the response letter, and text changes are highlighted in yellow in the revised manuscript. We now believe that our work would be more valuable to clinicians and researchers in the field.

Once again, we are deeply thankful for your time and efforts in reviewing our manuscript. We look forward to hearing from you about the status of our submission.

Yours sincerely,

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Editor’s Comments to Author

Editor:

1) May I ask you (i) to replace "et al" in the references list by the complete list of authors, and (ii) to add a separate list with the figure captions?

   (i) All the authors were added in the references as suggested;
   (ii) A separate list with the figure captions was added as suggested (at the end of the text).

2) It could be nice to have a scale bar on the graphical abstract (on the SEMs)

   The scale bar on the graphical abstract was added as suggested.

Reviewers’ Comments to Author

Reviewer #1:

Comments to Author

This study demonstrates the importance of sub-micron scale topography in directing MSC osteogenesis in vitro and bone formation in vivo. Furthermore, this study looks to explain these phenomena by investigating alterations in primary cilia and TGFB receptor localisation on these different topographies. The authors convincingly demonstrate that sub-micron topography drives bone formation, elongation of primary cilia and increased ciliary localisation of TGFB receptor II. Although these are very interesting findings in themselves, it has not been demonstrated that there is a functional link between these observed phenomena. A direct demonstration that alterations in cilia structure and protein localisation are required for osteogenesis would significantly add to this study.

General Comments

Although it is very intriguing that pTGBRII ciliary localisation increases with osteogenic differentiation, the justification for looking at TGF signalling is not clear. The authors have previously demonstrated altered Wnt signalling associated with topography induced changes in cilia length. Given that Wnt signalling is also associated with osteogenesis, this may prove a more fruitful path to take. Furthermore, the authors state that TGFB is associated with
osteogenesis. Although this is true at the very early stages of differentiation, it is known to inhibit later stage mineralisation.

As mentioned above, a functional link between the observations in this paper would significantly strengthen the paper. Have the authors considered investigating TGFβ signalling in their groups and determined whether this change in cilia length and receptor localization is correlated with actual change in TGFβ signalling and osteogenesis. Furthermore, recent work by Clement et al (fibroblasts) and Labour et al (hMSCs) have demonstrated other TGFβ signalling components localising to the cilium which could also be explored in this study.

Responses
The reviewer is absolutely correct that these interesting findings show a correlation between primary cilia expression, TGFβRII ciliary localization and osteogenesis in response to the two different calcium phosphate bone-substitute materials. We do not present direct evidence of a causal link. We agree that ciliary wnt signalling may also modulate osteogenesis and we have previously shown this to be influenced by changes in cilia length. The reviewer makes a good suggestion of additional experiments to measure nuclear β-catenin staining as an indicator of wnt signaling (as in our previous studies – McMurray et al). However we would prefer to keep the paper focused on TGF signaling rather than expand to investigate other potential ciliary mechanisms. In deed the cilia length changes associated with osteogenic materials may modulate a variety of cilia signaling pathways including wnt, hedgehog and mechanosignalling, all of which have been shown to influence osteogenesis. It is beyond the scope of the current study to examine all these potential mechanisms. Instead, we have further expanded the manuscript to discuss how topographically-induced changes in primary cilia expression may modulate osteogenesis through a variety of cilia signaling pathways. We have added additional references including those suggested by the reviewer (Discussion page 17-18).

We agree with the reviewer that TGFβ is associated with initial stages of differentiation whilst it may be inhibitory at later stages. We have now modified the text to clarify this throughout the manuscript. The differences in ciliary localization of TGFβRII in response to the two different
material surfaces was measured after 2-4 days of exposure to the material. Cilia length differences were still present at day 7. However the subsequent osteogenic differentiation was measured after 7 and 14 days in vitro and after 12 weeks in vivo. Therefore it is possible that early cilia elongation and recruitment of TGFβRII may lead to activation of TGFβ signaling promoting osteogenesis, but be followed by subsequent reductions in cilia length and downregulation of TGFβ signaling. Indeed prolonged TGFβ treatment is known to reduce cilia length consistent with such a feedback mechanism. We have expanded the text to discuss this interesting point raised by the reviewer (Discussion page 17).

Introduction:

(4, 1) showed should read shown.

This has been corrected.

(4, 2) A recent publication by Labour et al has been published demonstrating ciliary localisation of TGFβ signalling components in hMSCs and previous work by the Jacobs lab has demonstrated that cilia knockout in MSCs in vivo results in an inhibited loading induced bone formation. Thank the reviewer for providing the opportunity to reference these excellent additional study published recently by Labour et al. We apologize for missing this from the original submission. The work of Labour et al. supports the importance of MSC primary cilia in TGFβ signaling and the particular recruitment of other TGFβ signaling components. We have now included discussion of this nice paper in the manuscript (Discussion page 17).

Similarly, as suggested, we have added additional references from Chris Jacobs’ lab showing that MSC primary cilia are required for mechanically-induced bone formation and that changes in cilia length may influence mechanosignalling. Hence, biomaterial-induced alterations in cilia expression may effect bone deposition through altered mechanosignalling in addition to regulation of osteogenesis. (New references: [47] Hoey DA et al. Stem Cells. 2012, [48] Spasic and Jacobs, European Cells & Materials 2017) (Discussion page 17).

(4,2) TGFβ signalling is known to inhibit late stage differentiation in terms of mineralisation.

Please see response to ‘general comments’ above with resulting changes to the manuscript page 17-18.
Materials and Methods:

(6, 1) Removed 'was' in lines 1 and 2. The number of donors should be stated here also and details on the donors provided.

The text has been corrected and the details of the donors provided.

(8, 2) It is not clear what the seeding density was for comparison across groups. It would be better to write as cells/cm².

We have explained the seeding density in cells/cm² corresponding to the amount of the cells seeded as suggested.

Results:

(11, 3) How do the morphologies compare to cells cultured on glass slides. This should be included here as the data is there.

The cell morphology varied with the material surface, difference could be found in Figure 3A (as seen from the α-tubulin staining). The observation was included in 3.3. The α-tubulin staining indicated that the cell morphology on TCP discs and glass slice was different (Figure 3A). On TCP-S the cells were bigger in size than on TCP-B, while the cells on glass slice were more flattened as compared on TCP-S.

(12, 1) The restoration of cilia length on TCP-S is a very nice result. Can the authors discuss what the incidence did not recover. Was there differences in proliferation.

It is possible that the cells on TCP-B had reduced cilia incidence due to the lower confluence as shown in Figure 3A. This could modulate proliferation and actin tension, both of which influence ciliogenesis. We have added a sentence to note this possibility but have refrained from speculating on the mechanism.
Why choose a different timpoint to look at TGFBR localisation compared to cilia length? Also have teh authors considered looking at downstream cilia localisaed components suchs pR1 and pSMAD2/3.

We chose an early time point for the TGFβRII localization studies as TGF is involved in the early stages of differentiation as noted by the reviewer – please see response to earlier ‘general comments’ and associated changes in the manuscript.

It is an interesting suggestion to look at ciliary localization of other TGF components as has been done in the recent paper by Labour et al (see earlier comment). We have added reference to the Labour et al paper and explained how this provides a potential mechanism through which changes in cilia length may regulate TGF signaling (Discussion page 18).

Discussion:

The final statement in this paragraph might be a little overstated given teh lack of a functional link and analysis of TGFb signalling.

We have toned down the final conclusions regarding the potential topography-cilia-TGF-osteogenesis mechanism.

Reviewer #2:

The topic of this paper is very interesting and open new perspective for bone tissue engineering. However there is some points that have to be explained.

1. I don’t understand why authors use different concentration of cells 5000 or 10000 r 25000. For example 5000 to study cell morphology and 25 000 to study proliferation and différenciation. The number of cell could influence the results and has to be justified.

1) Cell density of 5, 000 cells and 25, 000 cells

Although different cell seeding densities were used, the cells responded to the material surfaces similarly according to the cell morphology analysis. The cell morphologies were clearly different between TCP-S and TCP-B on both cell densities of 5, 000 cells (Figure 2B) and 25, 000 cells (Figure 2A), the cells are larger and more spread on TCP-S than that on TCP-B.
The reason that we used different density is: the cell seeding density (5 000 cells) allowed us to analyse cell morphology and primary cilia expression of the single cells at day 1 and day 4, because cells were confluence and overlapping after 4-days cell culture when the cell seeding density is 25,000 cells, while the higher cell seeding density of 25,000 cells allowed us to have enough cells to run DNA, ALP and qPCR assays.

(2) Cell density of 5,000 cells (TCP discs) and 10,000 cells (discs)
In order to kept the similar cell density (±5000 cells/cm²) for both discs and cover slips, 5000 cells were seeded on ceramic discs (Ø=9 mm disc in 48-well plates) and 10 000 cells were seeded on cover slips (Ø=13 mm slip in 24-well plates).

2. I don’t understand why authors have used canine model to study ectopic bone formation. For ethic reasons, this seems to be criticable. The race of the dogs used must be given. Anaesthesia with pentobarbital is also criticable for dogs. Was there a breathing assistance?
For the ethic reason, it is quite difficult to run dog studies, while the dog model is the most often used model (and appeared to be most reliable model) to evaluate the osteoinductive potential of biomaterials [8]. Being the animal studies performed under approval of the local ethical committee and being them bred by an approved animal supplier, we could minimize ethical concerns. The race of the dogs is already specified in the submitted text, as “mongrel”. Anaesthesia via sodium pentobarbital without breathing assistance is a common method for this kind of simple surgery in dogs and the amount of sodium pentobarbital (30 mg/kg per body weight) is in the scope of the acceptable limits. In addition, following the surgeries, buprenorphine (0.1 mg per animal) was intramuscularly given to the animals as pain relief for 2 days, while penicilllin (40 mg/kg) was intramuscularly injected for 3 consecutive days to prevent infection.

3. Methods of histomorphometrical analysis have to be explained: number of sections per condition, software used, parameter analysed...
“Coronal” sections were made across the two transverse cuts (Figure 7A and B). All the sections were subjected to light microscopic observation with respect to bone formation. It turned out no bone was formed in TCP-B implant, but bone was formed in all TCP-S implants. One section across the middle of the TCP-S implant was subjected to quantification of bone per sample. The bone formation was quantified as the coverage (percentage) of the inner surface covered by bone (Outer surface was excluded because it is generally noted that material-driven bone formation was hardly seen on the outer surface of the implants). It is technically simple to measure the length of the inner surface and the length of inner surface covered by bone by ruler using the printouts of the scanned images. No special software was used.

4. In 3.5 Proliferation and osteogenic differentiation... there are some justifications of experimentation or materials and methods recall that should be in the specific sections of the paper.

Additional content about materials and methods in the result section 3.5 was removed as suggested.

5. Fig 6 How do you explain that osteocalcin gene expression decrease with time while osteocalcin is a late marker of osteodifferentiation. I have the same remark for ALP that increased with time while it is a precoce marker of osteodifferentiation.

As a late osteogenic differentiation marker, no significant difference was observed for OCN over time from day 4 to day 14. However, it appeared that the 3D culture of hBMSCs could promote the gene expression of OCN over time up to 14 days. We have shown that the gene expression of OCN was increased over time when hBMSCs cultured on porous TCP-B and TCP-S particles, with a highest OCN expression at day 14 [13]. It is possible that enhanced OCN gene expression would be expressed at late points after bone microtissues formation (e.g., day 21 or day 28) when the hBMSCs cultured on the 2D TCP discs (discussion page 16).

As the early osteogenic differentiation marker, ALP gene expression was up-regulated by TCP-S at day 7 in BM (the maximal ALP gene expression in BM in the experimental time). With the presence of dexamethasone (osteogenic medium), ALP gene expression was largely increased over time up to day 14. This result was in line with the literature data showing the increase of
ALP gene expression over time up to 14 days and the decrease thereafter in osteogenic medium [33]. We responded the comments in the discussion part (discussion page 15-16).

Reviewer #3:

**Paper Title:** Topography of calcium phosphate ceramics regulates primary cilia length and TGF receptor recruitment associated with osteogenesis.

This paper is a somewhat comprehensive paper trying to prove a hypothesis that the surface topography of calcium phosphate ceramics regulates stem cell morphology, primary cilia structure and TGF<beta> receptors recruitment to the cilium, associated with osteogenic differentiation. The subject of study is interesting that could have thrown some light but there are some concerns that remain to be addressed.

1. A major problem of this paper is lack of quantification of the surface topography. The materials were sintered at slightly different temperatures for the same duration. As a result, the grain sizes are not very different (e.g., a bit more than a micron and almost about micron). Pore sizes are not a lot different either. I believe that there is a synergistic effect between the grain size, pore size and pore volume, which makes some difference in the surface topography. But, it is not clear as to what extent the surface topography changes.

We did understand the concern of the reviewer. It is true that a slight difference in sintering temperature does not allow a big difference in surface topography if the materials were made from the same starting CaP powder. As we previously reported [12, 13] and also indicated in 2.1 (preparation of TCP-S and TCP-B ceramics), the surface topography of TCP-S and TCP-B was controlled by the reaction rate of calcium hydroxide and phosphoric acid. With a slow reaction, TCP-S could be obtained, while a quick reaction resulted in TCP-B. The different sintering temperature was used to adjust the microporosity (allowing similar microporosity between TCP-B and TCP-S) but not to control the surface topographic scale (from submicron to micron scale).
The reviewer is absolutely right. There is a synergistic effect among the grain size, pore size and pore volume, which makes the difference in the surface topography. By controlling the reaction rates, we controlled the grain size (Table 2, grain size), pore size (Table 2, pore size). By controlling the sintering temperature, we get the two materials with similar pore volume (Table 2, porosity). The surface topography, as commented by the reviewer, is a synergistic effect of grains, pores and pore volume.

*SEM and Hg-porosimetry have yielded some results, but quantification of the topography is absent. The experimental procedures for the determination of the grain size and pore size are poorly described. In view of the aforementioned comment, the rest of the data are not very meaningful.*

Next to the SEM images, we provide the physical properties of the TCP ceramics in Table 2. To show the clearly different surface topographies between TCP-B and TCP-S, we cited in the revision our published data of surface roughness of the two materials (Ra is 0.126±0.003um for TCP-S and Ra is 1.287±0.011um for TCP-B [12] (result part 3.1, page 11).

2. *It would be interesting if the author can provide data on the surface roughness of TCPs before and after soaking in BM. This can provide a better understanding of the underlying mechanism of differentiation.*

Since surface reaction between TCP ceramics and culture medium may occur, it is therefore interesting to see the possible change of surface topography once the materials were kept in culture for a long time (up to 14 days). Indeed, we reported the changes of porous TCP-B and TCP-S particles immersed into basic culture medium for 14 days [13]. It turned out that apatite could be formed in TCP-S but not TCP-B. However, the change of the materials in basic culture medium was so less that the charge could only be detected with XRD (X-ray diffraction) but not with SEM. In other word, *the change of surface topography of TCP-B and TCP-S after soaking in the culture medium was not detectable with SEM in the experimental setting (up to 14 days)* [13].
3. Would authors suggest any other mechanism that has synergic effect on promoting osteogenic differentiation than cilia elongation?

We have modified the discussion to describe how cilia elongation may be associated with altered wnt, hedgehog, and mechanosignalling as well as TGF signalling thereby modulating osteogenesis and bone formation (Discussion page 17). In addition, we also discussed that TCP-S may promote osteogenic differentiation through other mechanisms than surface topography as described by others [53], such as surface chemistry and ion release (discussion page 18).
Reference


Statement of significance

The surface topography of synthetic biomaterials plays important roles in material-driven osteogenesis. The data presented herein have shown that the surface topography of calcium phosphate ceramics regulates mesenchymal stromal cells (e.g., human bone marrow mesenchymal stromal cells, hBMSCs) with respect to morphology, primary cilia structure and TGFβR recruitment to the cillum associated with osteogenic differentiation *in vitro*. Together with bone formation *in vivo*, our results suggested a new type of biomaterial-based ciliotherapy for orthopedic, dental and maxillofacial surgery by the bioengineering control of osteogenesis via primary cilia modulation.
**Graphical Abstract**

- **TCP-B** and **TCP-S** comparison.
- MSCs differentiation in vitro and in vivo.
- Cell shape and cilia length analysis.
- ALP activity and DNA content.
- Bone apposition comparison.
Topography of calcium phosphate ceramics regulates primary cilia length and TGF receptor recruitment associated with osteogenesis

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Abstract

The surface topography of synthetic biomaterials is known to play a role in material-driven osteogenesis. Recent studies show that TGFβ signaling also initiates osteogenic differentiation. TGFβ signaling requires the recruitment of TGFβ receptors (TGFβR) to the primary cilia. In this study, we hypothesize that the surface topography of calcium phosphate ceramics regulates stem cell morphology, primary cilia structure and TGFβR recruitment to the cilium associated with osteogenic differentiation. We developed a 2D system using two types of tricalcium phosphate (TCP) ceramic discs with identical chemistry. One sample had a surface topography at micron-scale (TCP-B, with a bigger surface structure dimension) whilst the other had a surface topography at submicron scale (TCP-S, with a smaller surface structure dimension). In the absence of osteogenic differentiation factors, human bone marrow stromal cells (hBMSCs) were more spread on TCP-S than on TCP-B with alterations in actin organization and increased primary cilia prevalence and length. The cilia elongation on TCP-S was similar to that observed on glass in the presence of osteogenic media and was followed by recruitment of transforming growth factor-β RII (p-TGFβ RII) to the cilia axoneme. This was associated with enhanced osteogenic differentiation of hBMSCs on TCP-S, as shown by alkaline phosphatase activity and gene expression for key osteogenic markers in the absence of additional osteogenic growth factors.

Similarly, in vivo after a 12-week intramuscular implantation in dogs, TCP-S induced bone formation while TCP-B did not. It is most likely that the surface topography of calcium phosphate ceramics regulates primary cilia length and ciliary recruitment of p-TGFβ RII associated with osteogenesis and bone formation. This bioengineering control of osteogenesis via primary cilia modulation may represent a new type of biomaterial-based ciliotherapy for orthopedic, dental and maxillofacial surgery applications.

Keywords: calcium phosphate ceramic, topography, mesenchymal stromal cell, primary cilia, TGFβ, bone.
1. Introduction

Calcium phosphate (CaP) ceramics are widely used in orthopedic, dental and maxillofacial surgery as bone substitutes because of their chemical homology to native bone mineral, excellent biocompatibility and the ability to support osteogenesis on their surface (i.e. osteoconductivity) [1-3]. However, osteoinductivity of bone graft substitutes, i.e. the ability to positively induce osteogenic differentiation of stem cells to form bone, is required for bone regeneration in critical-sized bone defects [4, 5]. The most common approach to make CaP ceramics osteoinductive is to combine them with growth factors (e.g. bone morphogenic proteins, BMPs) [6]. However, the cost and safety of such approaches pose major concerns [7].

In the last decades, a subclass of CaP ceramics has been engineered to impart osteoinductivity without adding any osteogenic component, but only by tailoring their physico-chemical properties [8]. Among physico-chemical properties important for osteoinductivity, micropores (i.e. pores smaller than 10 µm) have long been recognized as a crucial material factor. Given the same chemistry, macroporous hydroxyapatite (HA) ceramics with micropores on their surface gave rise to bone formation following either subcutaneous [9] or intramuscular [10] implantation, while those without micropores failed. Similarly for CaP ceramics, the osteoinductive potential was also found to be correlated with microporosity such that higher microporosity resulted in greater osteoinductivity [11]. It has recently been shown that when two tricalcium phosphate (TCP) ceramics, having the same chemistry and microporosity were intramuscularly implanted, the one presenting submicron-scaled pores (0.65 ± 0.25 µm, TCP-S) induced ectopic bone formation while the other with micro-scaled pores (1.58 ± 0.65 µm, TCP-B) did not [12, 13]. The present study aimed to further examine the influence of surface topography on osteogenesis.

Despite several studies showing the influence of surface topography on cell differentiation both in 2D and in 3D cell culture systems [13, 14], the biological mechanism with which stem cells respond to surface structures and
undergo osteogenic differentiation remains unclear. Previous findings have indicated that substrate topography regulates cell morphology to control differentiation into specific lineages [14, 15]. For example, Guvendiren and Burdick showed that the size and pattern of surface wrinkles influenced MSCs morphology thereby regulating differentiation [16]. It has also been shown that cell morphology regulates MSC differentiation through mediation of RhoA activity without the requirement of soluble factors [17].

Primary cilia are single microtubule based hair like structures that respond to chemical and mechanical changes in the extracellular environment, coordinating multiple signaling pathways such as receptor tyrosine kinase (RTK), Hedgehog (Hh), Wnt, Notch, mTOR and mechanotransduction [18, 19]. It has been previously shown that primary cilia respond to changes in surface topography with MSC cilia elongation on grooved topographies [20]. This cilia response was mediated by changes in cell and actin morphology and was shown to regulate Wnt signalling. Furthermore primary cilia are required for osteogenic differentiation of MSCs [21] but how this regulation occurs is not known. It has been shown that recruitment of TGFR to the primary cilium is necessary for downstream TGF signalling [22] which is an important regulator of osteogenesis [23]. We therefore hypothesized that changes in surface topography of CaP ceramics may regulate cell morphology, primary cilia expression and ciliary recruitment of TGFR associated with osteogenesis.

To test this hypothesis, we investigated the morphology and primary cilia expression of hBMSCs on TCP ceramics with two distinct surface topographies (namely TCP-B and TCP-S). We investigated cell morphology, primary cilia expression, TGFR recruitment to the cilium and osteogenic differentiation in vitro and bone formation in an in vivo canine ectopic model. Here we show for the first time that hBMSCs grown on CaP ceramics with submicron surface topographies undergo osteogenic differentiation associated with changes in primary cilia structure and increased ciliary p-TGFBRII.
2. Materials and Methods

2.1 Preparation of TCP-S and TCP-B ceramics

TCP powders were prepared as previously described [13]. Briefly, a calcium hydroxide (Fluka) suspension and a phosphoric acid (Fluka) solution were mixed at a Ca/P ratio of 1.50. TCP-S and TCP-B powders were obtained by controlling the respective reaction rates. The green bodies were then obtained after mixing the TCP-S and TCP-B powders with diluted H₂O₂ (0.1%) (Merck). The TCP-S and TCP-B ceramics were finally achieved by sintering the dry green bodies at 1050 °C (TCP-S) and 1100 °C (TCP-B) for 8 hours, respectively.

TCP-S and TCP-B discs (Φ9 x 1mm) were machined using a diamond-coated saw microtome (SP-1600, Leica, Germany) for in vitro evaluation. Ceramic cylinders (Φ9 x 12mm) with two transverse cuts of 1.1 ± 0.1mm were made as well for in vivo evaluation (Figure 1A). The obtained materials were then ultrasonically cleaned with acetone, 70% ethanol and demineralized water, and dried at 80 °C. All samples were steam sterilized at 121 °C for 30 min and dried at 80 °C afterwards.

Crystal chemistry of the TCP-S and TCP-B ceramics were determined with X-ray diffraction (XRD, Rigaku, Japan) and confirmed to be β-TCP. Surface morphology was observed with an environmental scanning electron microscope (ESEM; XL30, ESEMFEG, Philips, Eindhoven, The Netherlands) in the secondary electron mode; at the same time, grain size and pore size were measured with 10 images at the magnification of 5000. Porosity, pore distribution and total pore area were determined by mercury intrusion testing (Micromeritics, USA).

2.2 In vitro cell culture

2.2.1. Isolation and expansion of hBMSCs

hBMSCs from three donors were isolated from bone marrow aspirates with as previously described [13, 24, 25]. In brief, aspirates from the donors were re-suspended using a 20 G needle, plated at a density of 5×10⁵
cells/cm² and cultured in proliferation media (PM) for expansion. **PM consisted** of basic media (BM) and basic fibroblasts growth factor (bFGF, Instruchemie, the Netherlands, 1ng/mL). BM was consisted of alpha-MEM (Life Technonologies) supplemented with 10% of fetal bovine serum (FBS, Life Technologies), 0.2 mM ascorbic acid (ASAP, Life Technologies), 20 mM L-glutamine (Life Technologies), 100 U/mL penicillin (Life Technologies) and 100 µg/mL streptomycin (Life Technologies). Cells were grown at 37 °C in a humid atmosphere with 5% CO₂, media that was refreshed twice per week and cells were sub-cultured when they reached 80-90% confluency. Passage 2-3 hBMSCs were used.

**2.2.2 Cell culture on TCP ceramics**

To study the effect of surface topography on cellular behavior, hBMSCs were cultured on the TCP discs. All the discs were placed in non-treated 48-well plate and soaked in BM for at least four hours before cell seeding. To evaluate cell morphology (actin staining) and primary cilia expression, cells were seeded onto the TCP discs at a density of 5,000 cells/cm². For cell attachment, SEM analysis of morphology, cell proliferation, osteogenic differentiation, gene expression and analysis of ciliary p-TGFβR II, cells were seeded at a density of 25,000 cells/cm² per disc in 1 mL basal media (BM). Additional studies were conducted in the presence of osteogenic media (OM) containing 10⁻⁸ M dexamethasone in addition to BM composition for gene expression. Cells were cultured on ceramic discs at 37 °C in a humid atmosphere with 5% CO₂. The media was refreshed twice per week.
2.2.3. SEM analysis of cell attachment and morphology

For cell attachment and morphology observation, cells on TCP discs were viewed at day 1 with methylene blue staining and SEM observations. After fixing with 4% paraformaldehyde and washing with PBS, the samples were stained with 1% methylene blue and viewed with a steromicroscope (LM; E600, Nikon SMZ-10A, Japan). Thereafter, the samples were dehydrated in sequential ethanol series and followed by critical point drying from liquid carbon dioxide using a Balzers CPD 030 Critical Point Dryer. The samples were gold sputter coated (Cressington) before being imaged by SEM.

2.2.4. Actin staining and cell morphology analysis

To analyze cell morphology individually, cells (5000 cells/cm², n=3 per condition) were cultured on TCP ceramics for day 1 and day 4 and then fixated for 30 minutes in 4% paraformaldehyde, washed with PBS and permeabilised in 0.25% Triton-X 100 in PBS. Subsequently, F-actin was stained using phalloidin-AF488 (LifeTechnologies) and nuclei were stained using DAPI (Sigma Aldrich). After washing with PBS, montage images were captured using a BD Pathway system (BD Pathway 435, BD biosciences). The image analysis program Cell Profiler was used to quantitatively measure the morphological characteristics of cells cultured on TCP-B and TCP-S surface. Depicted descriptors for cellular morphology were chosen based on relevance and statistically significant differences between TCP-S and TCP-B. Such measures included the cell area and the form factor (ratio of minor over major axis lengths).

2.2.5. Confocal microscopy analysis of primary cilia expression and TGFR localisation

To evaluate the effect of surface topography on primary cilia occurrence and length, cells (5,000 cells/cm², n=3 per condition) cultured on TCP ceramics up to 7 days underwent serum starvation for 24 hours before fixation. Cells were fixed with 4 % paraformaldehyde at 37 °C for 10 minutes, permeabilised in 0.5% Triton and blocked
with 5% goat serum. Primary cilia were labeled using anti-acetylated α-tubulin antibody (clone 6-11 B-1, 1:2,000; Sigma-Aldrich) and pericentrin antibody (Abcam, ab448-100) at 4 °C for overnight, washed thereafter and incubated for 1 hour at 25 °C with Alexa 488 Anti-mouse conjugate and Alexa Fluor 594 F(ab’)2 fragment of goat anti-rabbit IgG (H+L) (Invitrogen). p-TGFβR II was labelled using p-TGFβR II (Tyr-424) Santa Cruz Biotechnology, Sc-17007-R and Alexa Fluor 633 goat anti-rabbit IgG (H+L). Finally, the samples were mounted with a DAPI counterstaining (Invitrogen). Maximal projections of confocal z-stacks were created with a Leica SP2 confocal microscope (pixel size = 0.1 μm). Cilia prevalence was assessed based on the percentage of ciliated cells per field of view (n=5 fields), cilia length and ciliary p-TGFR II intensity were measured using image J software as previously described [20, 26].

Additional studies were conducted to determine the effect of OM on primary cilia expression and length. For these studies, hBMSCs were cultured on glass coverslips (Ø13mm) in 24-well plates in either BM or OM. Cells were seeded at a density of 5000 cells/cm² per well with media refreshed at day 4. Cells were serum starved for 24 hours prior to fixation at day 7 for analysis of primary cilia expression.

2.2.6. Cell proliferation and ALP assays

For cell proliferation and ALP assays, samples (3 donors, 25,000 cells/cm², n=3 per condition) were harvested at day 1, day 4, day 7 and day 14 respectively. The samples were gently rinsed three times with PBS, dried by aspirating PBS, and stored at -20 °C until further use. 500 μL of lysis buffer (prepared according to manufacturer’s instructions of CyQuant Cell Proliferation Assay kit instructions) was added onto each sample, followed by three cycles of freezing and thawing at -20 °C and room temperature, respectively. Cell proliferation was analyzed with a DNA assay (CyQuant Cell Proliferation Assay kit, Sigma, the Netherlands), according to the manufacturer’s instruction. Briefly, 100 μL cell lysate and DNA standard were incubated with 100 μL CyQuant GR dye at room temperature for 15 minutes and measured using a spectrophotometer (Victor, Perkin Elmer) at an excitation wavelength of 480 nm and emission wavelength of 520 nm. ALP activity was
measured using a CDP-star assay kit (Roche). 40 µL of CDP star substrate were incubated with 10 µL cell lysate for 20 min, after which the luminescence was measured using a spectrophotometer (Victor, Perkin Elmer). ALP expression was normalized to DNA content.

To examine the osteogenic differentiation of cells on TCP discs, ALP staining was conducted on the 7-day samples, following the manufacturer’s protocol of the Alkaline Phosphatase kit (Sigma-Aldrich). Samples were first washed three times with PBS, fixed with 4% paraformaldehyde for 30 seconds, and then incubated for 30 minutes in staining solution containing Naphtol AS MX phosphate and Fast Blue. The ALP positive cells were stained blue. Finally, the samples were washed 3 times with deionized water and observed under the stereomicroscope (LM; E600, Nikon SMZ-10A, Japan).

2.2.7. PCR analysis of osteogenic gene expression

For gene expression, cells were cultured on TCP discs in both BM and OM. Samples (n=3 per condition) were collected at day 4, day 7 and day 14. Bone-related gene expression was evaluated with quantitative real-time polymerase chain reaction (PCR) assay. RNA isolation was performed using Trizol reagent (Invitrogen) and Nucleospin RNA isolation kit (Macherey-Nagel GmbH & Co.) according to the manufacturer’s instructions. Total RNA was measured using a NanoDrop spectrophotometer (Nanodrop technologies, USA). The RNA was used to synthesize complementary DNA (cDNA) with an iScript cDNA Synthesis kit (BioRad) according to the manufacturer’s instructions. PCR analysis was performed with a Bio-Rad real-time PCR system (Bio-Rad, Hercules, CA, USA) on alkaline phosphate (ALP), collagen type I (Col I), osteocalcin (OCN), and osteopontin (OPN), with beta-2 microglobulin (B2M) as the house-keeping gene used for normalization. Primer sequences for ALP, Col I, OCN, OPN, and B2M are listed in Table 1. The relative amounts of target genes normalized by B2M were calculated by $2^{\Delta C_T}$ method where $\Delta C_T=C_{T,\text{Target}}-C_{T,\text{B2M}}$.
2.3. *In vivo* bone formation assay

TCP-B and TCP-S samples were implanted in an ectopic canine model for 12 weeks. Following the permission of the local animal care committee (Animal Center, Sichuan University, Chengdu, China), the TCP-S and TCP-B cylinders (Figure 1A) were implanted in the para-spinal muscles of 8 adult male dogs (mongrel, 10-15 kg). All surgeries were conducted under general anaesthesia by abdominal injection of sodium pentobarbital (30 mg/kg body weight) and sterile condition. Following the surgeries, buprenorphine (0.1 mg per animal) was intramuscularly given to the animals as pain relief for 2 days, while penicillin (40 mg/kg) was intramuscularly injected for 3 consecutive days to prevent infection. After operation, the animals were allowed for full weight bearing and received normal diet. After 12 weeks, the dogs were sacrificed by a celiac injection of excessive amount of pentobarbital sodium. Implants were harvested with surrounding tissues and fixed in 4% formaldehyde, and embedded in poly (methyl methacrylate) (PMMA) after a series of gradient ethanol dehydration. Non-decalcified sections were prepared trans-crossing the transverse cuts using a diamond saw (SP-1600, Leica, Germany) and stained with 1% methylene blue (Sigma) and 0.3% basic fuchsine (Sigma) solutions. Histological observations were performed using light microscopy to evaluate bone formation in the explants. The histological slide crossing the middle of each explant was scanned with a scanner (DIMAGE Scan Elite5400 II, model AF5400-2, KONICA MINOLTA). **Inner surface of the transverse cuts and the inner surface covered by bone were measured in length with the printouts of the scanned images.** Bone formation was quantified as coverage of the inner surface [%].

2.4. Statistical analysis

Quantitative results are shown as average ± standard deviation. Multiple comparisons were performed with two-way analysis of variance (ANOVA) followed by Bonferroni post-test comparisons. P-values lower than 0.05 were considered as statistically significant differences.
3. Results

3.1. Characterization of TCP-S and TCP-B ceramics

Figure 1A shows the images of TCP samples for both in vitro and in vivo evaluation. XRD analysis revealed that the two TCP ceramics had the same chemistry of β-TCP (Figure 1B). TCP with different sizes of surface microstructure was prepared shown by SEM; TCP-B contained larger grains and micropore size than those of TCP-S leading to differences in surface topography (Figure 1C, D). Grain size, pore size, porosity, and total pore surface area were summarized in Table 2. The unique grains and pores in TCP-S and TCP-B resulted in different surface roughness in the two materials, with the Ra of 0.126±0.003 µm for TCP-S and 1.287±0.011 µm for TCP-B as reported in our previous study [12].

3.2. hBMSC morphology and attachment is regulated by TCP surface topography.

Cells were found to be homogeneously distributed on both TCP discs after 24 hours (Figure 2A, methylene blue staining images). SEM images showed clear attachment of hBMSCs to the TCP substrate and confirmed that cells were larger and more spread on TCP-S than on TCP-B (Figure 2A). Fluorescent imaging of F-actin showed that cells cultured on TCP-S were more spread and had larger area compared to those on TCP-B after 1 and 4 days (Figure 2B). The morphological change was similar at early time on TCP-S and TCP-B between the low cell seeding density (5000 cells/cm², Figure 2B) and the high cell seeding density (25000 cells/cm², Figure 2A). The quantitative data confirmed that the cells on TCP-S had significantly larger area, but similar form factor, compared to those on TCP-B (Figure 2C). The form factor values of cells ranged between 0.3 and 0.4 for both TCP ceramics, suggesting that cells had elongated morphology on both materials. However, those on TCP-S were more spread than on TCP-B, as shown by all the other morphological indicators, and had a greater level of F-actin staining. Evidently, the size of the surface microstructure influenced the morphology and cytoskeletal organisation of hBMSCs cultured on TCP discs for 1 and 4 days.
3.3. Primary cilia elongation occurs in response to TCP-S topography and osteogenic media

The α-tubulin staining indicated that the cell morphology on TCP discs and glass slice was different (Figure 3A). On TCP-S the cells were bigger in size than on TCP-B, while the cells on glass slice were more flattened as compared on TCP-S. As shown in Figure 3A and 3B Primary cilia expressed by hBMSCs cultured for 7 days on both glass coverslips and TCP ceramic surfaces were fluorescently labeled for acetylated alpha tubulin and pericentrin and imaged using confocal microscopy (Figure 3A). On glass coverslips, approximately 90-100% of hBMSCs expressed primary cilia although this incidence was slightly reduced by the addition of dexamethasone present in the OM (Figure 3B) (p<0.05). However osteogenic media significantly increased the length of primary cilia from a mean value of approximately 2.6 μm to 3.5 μm (Figure 3C, p<0.001). Fewer primary cilia were present in cells cultured on TCP ceramics compared to glass in either culture media, with the prevalence on TCP-B being particularly low, possibly due to the fact that these cells appeared less confluent (Figure 3A and B). Both primary cilia prevalence and length were affected by TCP ceramic surfaces (TCP-S vs TCP-B). In particular, culturing cells on TCP-S induced a significant increase in cilia prevalence and length (Figure 3B, p<0.01), compared to that observed on TCP-B (Figure 3C, p<0.001). Primary cilia of cells cultured on TCP-S in BM were equivalent in length to those expressed by cells cultured on glass in osteogenic media with dexamethasone.

3.4. Primary cilia recruitment of p-TGFβ RII is increased on TCP-S surfaces

Ciliary length and p-TGFβ RII levels were investigated at day 2 and day 4 of culture by confocal immunofluorescent imaging of acetylated alpha tubulin and p-TGFβ RII (Figure 4A). By day 4, cilia prevalence was significantly greater on TCP-S ceramics compared to TCP-B (Figure 4B) reflecting the difference observed at day 7 (Figure 3B). Culturing cells on TCP-S caused cilia elongation at both day 2 and day 4 compared to cells on TCP-B, with greatest cilia length observed at day 2 (Figure 4C). Localisation of p-TGFβ RII on the cilia axoneme was higher in hBMSCs grown on TCP-S than on TCP-B at both day 2 and day 4, the difference being statistically significant by day 4 (Figure 4D, p<0.001).
3.5. Proliferation and osteogenic differentiation of hBMSCs are enhanced on TCP-S surfaces

Cells from 3 donors (n=3) displayed increased proliferation over time from day 1 to day 14 on TCP-S, while cell proliferation on TCP-B varied with donors. Cells from one donor did not display any difference in proliferation rate on TCP-B over the time course studied. Enhanced cell proliferation on TCP-S was observed in hBMSCs from all the donors, two of them being significantly higher compared to the cells grown on TCP-B (Figure 5A, left panel).

ALP production varied among the donors (Figure 5A, right panel). In particular, ALP activity slightly decreased over time from day 4 to 14 in cells from donor 1 and 2 on both TCP ceramics, while those from donor 3 did not change their ALP activity with time from day 4 to 14. When TCP-S and TCP-B were compared, significantly higher ALP activity on TCP-S than on TCP-B was observed (Donor 1: at day 7 and 14; Donor 2: at day 7 and 14; donor 3: at day 7). ALP immuno-staining of a set of 7-day samples displayed increased ALP activity on TCP-S (Figure 5B).

3.6. Osteogenic gene expression is enhanced on TCP-S surfaces

Col I expression was downregulated in BM over time on TCP-S, while there was no significant change in hBMSCs cultured on TCP-B. ALP gene expression was up-regulated in BM from day 4 to 7 and slightly down-regulated from day 7 to 14 on TCP-S. There was no significant difference of ALP gene expression on TCP-B in BM between day 4 and day 7, but it was slightly increased at day 14. Down regulation of OCN in BM was observed on both TCP ceramics from day 7 to day 14, but not significant. The most significant up-regulation in BM was observed for OPN on TCP-S, which was 4 times higher at day 7 and 10 times higher at day 14 compared to day 4, but only slightly up regulated (less than 2 times) from day 4 to day 14 on TCP-B. Comparing the cells on TCP-S and TCP-B, Col I expression in BM was significantly higher on TCP-S at day 4 and 7. ALP gene expression in BM was approximately three times higher on TCP-S at day 7 compared to TCP-B. OCN
expression in BM was also enhanced on TCP-S at day 4, 7 and 14 compared to that observed on TCP-B. OPN expression displayed the most striking difference amongst osteogenic markers analysed in BM on TCP-S and TCP-B. It was significantly increased on TCP-S, being three times higher at day 7 and five times higher at day 14 compared to that observed on TCP-B.

Comparing osteogenic gene expression of hBMSCs cultured on TCP discs in BM and OM revealed that dexamethasone in OM exerted a down-regulatory effect on Col I, OCN and OPN expression, but up-regulated ALP expression. In OM, TCP-S enhanced Col I gene expression at day 4 and 7, and OCN and OPN gene expressions at day 14. The enhancement of ALP gene expression on TCP-S in BM disappeared in OM.

3.7. TCP-S induces greater bone formation in vivo.

In total, 8 samples per TCP ceramic were harvested from 8 dogs for histological evaluation and histomorphometry. No bone formation was observed in any of the TCP-B samples (Figure 7A, C, E), while bone was formed in 7 out of 8 TCP-S explants (Figure 7B, D, F). Images with high magnification showed the presence of bone on the inner surface of TCP-S samples (Figure 7F) and only soft tissues were observed in TCP-B (Figure 7E). Quantitatively, 28 ± 17% of the available inner surface of TCP-S explants was occupied by bone.

4. Discussion

Chemical design of materials and application of biological molecules are often used to achieve specific biological responses in tissue regeneration. However there is an increasing amount of evidence suggesting that physical, mechanical or topographical properties of biomaterials also play a pivotal role in controlling biological functions [27]. In particular, it has been reported that the micro- and nano-structured surfaces of biomaterials can mediate cellular behavior including adhesion, morphology, proliferation and differentiation in vitro [28, 29]. Here, we reported that surface structure or topography of TCP ceramics affect hBMSC morphology, primary
cilia expression and ciliary recruitment of p-TGFβR II in vitro. Furthermore, these differences were associated with regulation of osteogenesis. Thus submicron scaled surface features (TCP-S) induced greater cell spreading (Figure 2), increased primary cilia expression, cilia elongation and recruitment of p-TGFβR II into the cilium (Figures 3 and 4) associated with increased osteogenic differentiation at both protein (Figure 5) and gene level (Figure 6). Furthermore, following an ectopic implantation, this material (TCP-S) also gave rise to heterotopic bone formation in muscle while TCP ceramic implants with micron scaled surface structure (TCP-B) did not (Figure 7). These findings suggest for the first time, that the topographical cues may drive osteogenic differentiation by modulating primary cilia structure and ciliary recruitment of p-TGFβR II, which is required to activate TGFβ signaling.

The implantation of materials in vivo always follows the wound healing processes inherent of the innate immune reaction, followed by angiogenesis, tissue formation and remodeling. Many cell types (e.g. macrophages, myoblasts, MSCs and pericytes) involved in wound healing may be sensitive to surface structures and contribute to tissue morphogenesis. In the case of ectopic bone formation, osteogenic differentiation of MSCs infiltrated into the implants is crucial to induce bone regeneration [8]. A previous study has also demonstrated that BMSCs were involved in inductive bone formation in osteoinductive CaP ceramics [30]. Hence, our in vitro studies focused on the response of hBMSCs to the surface structure of TCP ceramics osteogenic differentiation.

The induction of stem cell osteogenesis by surface structure is dependent on the geometry and size of the surface structure, its spatial organization and the dynamical changes of the surface properties during time [31].

Col I, ALP, OCN and OPN genes are pivotal factors in osteogenic differentiation, matrix deposition and mineralization [13, 31, 32]. Without the presence of osteogenic factors, TCP-S not only promoted ALP activity but also up-regulated all bone-related gene expression tested in this study (Figure 6). As the early osteogenic differentiation marker, ALP gene expression was slightly up-regulated by TCP-S at day 7 in BM (the maximal
ALP gene expression in BM in the experimental time). With the presence of dexamethasone (osteogenic medium), ALP gene expression was largely up-regulated and increased with time up to day 14. This result was in line with the literature data showing the increase of ALP gene expression up to 14 days and the decrease thereafter in osteogenic medium [33]. No up-regulation of OCN over time from day 4 to day 14 was observed in this study, it might be possible that the enhanced gene expression of OCN could be expected at the late time points (e.g. day 21 or day 28) since OCN is a late osteogenic differentiation marker [33]. Given the fact that no growth factors were used, the gene expression in BM indicates that the dimension of surface structure alone could instruct osteogenic differentiation of hBMSCs. This result is in line with our previous data showing osteogenic regulation by the surface structure in 3D TCP ceramic granules [13].

The reaction of stem cells to the surface structure of the biomaterials is one of the first steps required for osteogenic differentiation induction [34]. The possibility of cells to make successful protrusions and contacts in a given direction varies with the size of the surface feature [35]. In other words, cells adapt their morphology according to the surface topography of the substrates they are cultured on [36]. It is known that cell differentiation is often associated with the morphological changes of the cells [17, 37]. For instance, when hBMSCs were cultured on substrates with adhesive islands of various sizes, changes in cell shape that occurred depending on the space available was responsible for hBMSC commitment to either adipogenic or osteogenic lineages [17, 38]. In particular, hBMSCs with rounded morphology differentiated into adipose cells, while hBMSCs with more spread morphology underwent osteogenic differentiation. The same correlation between cell morphology and osteogenic differentiation of hBMSCs was also seen in response to TCP ceramics in this study. Cells on both TCP-S and TCP-B were elongated, but those on TCP-S were more spread (Figures 2) and underwent greater osteogenic differentiation (Figures 5 and 6).

Changes in cell morphology have been associated with alterations in intracellular cytoskeletal tension leading to altered expression of integrins and cadherins [17, 39] and primary cilia structure [40]. Recent findings show
that cytoskeletal modulation by substrate topography affects primary cilia structure thereby regulating Wnt signalling in hBMSCs [20]. Furthermore primary cilia have been shown to be required for hBMSC osteogenic differentiation [21] and to undergo cilia elongation during osteogenesis as confirmed in the present study (Figure 3C) [26, 41]. These previous studies prompted the question as to whether the osteoinductive properties of TCP-S ceramics are associated with changes in primary cilia structure and function resulting from the alterations in cell morphology.

Previous reports have shown that TGFβ can induce osteogenic differentiation in hBMSCs [23]. In addition, TGFβ signalling is regulated by substrate stiffness and cytoskeletal tension, although the underlying mechanisms are unknown. Studies from Christensen’s group have shown that primary cilia regulate TGFβ signalling in fibroblasts associated with the recruitment of TGFβ receptors to the cilia axoneme [22]. Furthermore Hoey’s group have also shown how the receptors and downstream components in TGFβ signalling are localised to primary cilia in mesenchymal stem cells associated with activation of SMAD3 at the ciliary base [42]. In the present study, we observed that primary cilia length was increased on osteoinductive TCP-S ceramics and that this was associated with increased ciliary p-TGFβ RII. The time lag between initial cilia elongation and TGFβRII ciliary localisation may reflect slightly different intraflagellar transport (IFT) dynamics/mechanisms governing the movement of tubulin and TGF receptors onto the axoneme. The increased ciliary localization of TGF receptors, as seen on TCP-S ceramics, could provide a specialized environment to increase interactions between TGF pathway components hence causing increased TGFβ pathway activation that is essential in initiating osteogenic differentiation of hBMSCs. Our data therefore suggest for the first time, that surface topography regulates primary cilia structure and associated TGFβ RII localisation in the cilium, thereby enabling TGFβ signalling which is necessary for osteogenic differentiation of hBMSCs. However, it should also be noted whilst TGFβ signalling drives early osteogenesis, it is inhibitory at later stages. Interestingly we have shown previously that prolonged TGFβ causes primary cilia shortening [26], which may therefore act as a feedback mechanism down modulating TGFβ signalling and osteogenesis at later stages.
In addition to regulation of TGFβ signalling, primary cilia also regulate a number of other osteogenic signaling pathways, such as Wnt [20, 43], runx2 [44], and hedgehog [45, 46]. Indeed, previous studies from Knight's group have shown that changes in primary cilia length can modulate both hedgehog and wnt signaling pathways [20, 42, 44, 45]. Furthermore, in mesenchymal stem cells, primary cilia are necessary for mechanosignalling [47] which also regulated differentiation. Other recent studies report that cilia elongation increases mechanosignalling in bone [48]. Therefore changes in cilia length in response to osteogenic TCP topography may modulate in vivo bone formation through altered mechanosignalling in addition to regulation of osteogenesis.

Finally, dexamethasone is often used as an osteogenic factor, because it is essential for the full differentiation of hBMSCs into mineral-producing osteoblasts as shown by increased ALP activity [49, 50]. However, in the present study, hBMSCs cultured in OM with dexamethasone on TCP ceramics showed reduced levels of Col I, OCN, and OPN gene expression compared to cells in BM. These data suggested that ALP expression could be uncoupled from Col I, OCN and OPN, in agreement with previous studies showing that dexmethasone inhibited or postponed Col I and reduced OCN expression [51, 52]. Interestingly the surface structure of the osteo-inductive TCP-S ceramics had a similar effect on primary cilia length with that induced by dexamethasone in the osteogenic media for cells cultured on glass (no difference in cilia length between OM Glass vs BM TCP-S, Figure 4C). This further highlights the potential potency of topography and cilia modulation as a means of controlling osteogenesis.

In addition to direct topography induced cilia elongation, it cannot be ruled out that changes in topography may influence the local surface chemistry and local ion release which may impact on osteogenesis as previously described [53]. It has been shown that a apatite layer was formed on the surface of TCP-S without detectable surface structure change when TCP-S was contacted to body fluids (e.g. culture medium) [13].
5. Conclusion

In this study we compared tri-calcium phosphate (TCP) ceramics with micron or sub-micron scaled surface structure, termed TCP-B and TCP-S respectively. TCP ceramic with a submicron scale surface induced a more spread stem cell morphology, increased expression and length of primary cilia, recruitment of p-TGFβ RII to the ciliary axoneme and osteogenic differentiation at a cellular and molecular level without any additional osteogenic factors in vitro. Furthermore, this osteogenic response was associated with increased inductive bone formation in vivo. These data not only highlight the importance of topography in regulating osteogenesis but also imply a novel mechanism involving primary cilia elongation and recruitment of p-TGFβ RII to the ciliary axoneme. This may therefore represent a new biomaterial based ‘ciliotherapy’ for use in orthopedic, dental and maxillofacial surgery applications.

6. Acknowledgements

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7. Contributions

All primary cilia and TGFβ experimental work was conducted by MD with support from PC and MK at Queen Mary University of London. The preparation and characterization of TCP, the in vitro analysis of ALP and osteogenic gene expression was conducted by JZ with support from XL, EV, DB, LM and HY at Maastricht.
University. In vivo analysis was conducted by HY at Sichuan University. JB, CB provided ideas. JZ, MD, MK, and HY conceived the study and wrote the paper. All authors were involved in analysis of different aspects of the results.
8. Reference


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Figures Legends and table captions

Figure 1. TCP ceramics were created with identical chemistry but different surface topography as shown by XRD and SEM respectively. Images of samples used for in vitro and in vivo evaluations (A); chemistry of TCP ceramics analyzed with XRD (B); SEM images of TCP-B (C) and TCP-S (D).

Figure 2. The morphology and actin organization of hBMSCs is regulated by the surface topography of TCP ceramics. A: Methylene blue staining of and SEM observation of hBMSCs cultured on TCP-B and TCP-S for 24 hours; B: Representative images of cell nucleus (DAPI) and actin skeleton (phalloidin) of hBMSCs on TCP discs for 1 and 4 days; C: The area and form factor plot of hBMSCs cultured on TCP-S and TCP-B for 1 and 4 days.

Figure 3. Primary cilia expression and length is modulated by osteogenic differentiation media and TCP topography. Confocal images showing primary cilia labelled with acetylated α-tubulin (green) in hBMSCs cultured on glass in either BM or OM and on TCP ceramic surfaces in BM for 7 days (A). Scale bars are 10 μm for field images and 3 μm for boxed images of single cilium. Cilia basal bodies are labelled with pericentrin (red) and nuclei with DAPI (blue). Corresponding primary cilia prevalence (n=10 fields of view) (B) and length (n>100 cilia) (C) (* p< 0.05, ** p< 0.001, ***p< 0.0001 for OM vs. BM and for TCP-S vs. TCP-B; $^+$ p< 0.05, $^{++}$ p< 0.001, $^{+++}$ p< 0.001 for TCP vs. BM; $^#p< 0.05, ^{##}p< 0.001, ^{###}p< 0.0001$ for TCP vs. OM), Mann-Whitney test.

Figure 4. hBMSCs grown on TCP-S surfaces have longer cilia and increased ciliary p-TGFB RII over four days of culture. (A) Representative images of primary cilia labelled for acetylated α-tubulin (left, green) of hBMSCs cultured on TCP-B and and TCP-S surfaces at day 2 and day 4 of culture with corresponding images labeled for p-TGFB RII (middle, red). Overlaid images were shown at right. Scale bars 2 μm. (B) Cilia prevalence, (C) length and (D) mean ciliary p-TGFB RII intensity for each group as described above for day 2 and day 4. n =
100-110 cilia measured per group for (C) and (D), n ≥ 5 fields per group with ≥ 15 cells/field for (B). *: p<0.05, ***: p<0.001, TCP-S against TCP-B at correlating time point.

Figure 5. hBMSCs cultured on TCP-S show increased proliferation and osteogenic differentiation compared to cells on TCP-B. DNA quantification and ALP activity (normalized to DNA amount) of hBMSCs from 3 donors cultured on TCP discs over a 14-day period (A). Representative images of hBMSCs cultured for 7 days on TCP surfaces and showing more intense blue ALP staining on TCP-S (B). *: p<0.05, **: p<0.005, ***: p<0.001, TCP-S against TCP-B at correlating time point.

Figure 6. hBMSCs cultured on TCP-S show increased osteogenic gene expression compared to cells on TCP-B. Osteogenic genes expression profile of hBMSCs on TCP-S and TCP-B discs and in both BM and OM normalized to the B2M (housekeeping gene). *: p<0.05, **: p<0.005, ***: p<0.001, Mann-Whitney test.

Figure 7. Inductive bone formation around TCP-S but not TCP-B in canine in vivo model. Histological overviews of TCP-B (A, C) and TCP-S (B, D) samples after a 12-week implantation in muscle of dog, showing bone formation in TCP-S and no bone in TCP-B; a high magnification image of TCP-B (E) explants, showing the infiltration of fibrous tissue but absence of bone in TCP-B; a high magnification image of TCP-S (F) explants, showing the presence of mineralized bone matrix (bright pink). (M: materials; B: bone; ST: soft tissue)

Table 1 qPCR primer sequences

Table 2 Physical properties of TCP ceramics
Figure 1

A) In vitro and In vivo samples of TCP ceramics.

B) Chemistry of TCP ceramics showing X-ray diffraction patterns for TCP-S and TCP-B.

C) Scanning electron micrograph (SEM) of TCP-S showing a porous structure at 10 μm scale.

D) SEM of TCP-B showing a more compact structure at 10 μm scale.
Figure 2
Figure 5

Click here to download high resolution image

A

Donor 1

DNA

ALP activity

TCP-B
TCP-S

Donor 2

DNA

ALP activity

TCP-B
TCP-S

Donor 3

DNA

ALP activity

TCP-B
TCP-S

B

Day 7

TCP-B

TCP-S

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