RALA complexed α -TCP nanoparticle delivery to mesenchymal stem cells induces bone formation in tissue engineered constructs *in vitro* and *in vivo*

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Abstract

A range of bone regeneration strategies, from growth factor delivery and/or mesenchymal stem cell (MSC) transplantation to endochondral tissue engineering, have been developed in recent years. Despite their tremendous promise, the clinical translation and future use of many of these strategies is being hampered by concerns such as off target effects associated with growth factor delivery. Therefore the overall objective of this study was to investigate the influence of alpha-tricalcium phosphate (α -TCP) nanoparticle delivery into MSCs using a RALA cell penetrating peptide on osteogenesis *in vitro* and both intramembranous and endochondral bone formation *in vivo*. RALA conjugated α -TCP nanoparticle delivery to MSCs resulted in an increased expression of bone morphogenetic protein-2 (BMP-2) and an upregulation in a number of key osteogenic genes. When α -TCP stimulated MSCs were encapsulated into alginate hydrogels, enhanced mineralization of the engineered construct was observed over a 28 day culture period. Furthermore, the *in vivo* bone forming potential of RALA conjugated α -TCP nanoparticle delivery to MSCs was found to be comparable to growth factor delivery. Recognizing the potential and limitations associated with endochondral bone tissue engineering strategies, we then sought to explore how α -TCP nanoparticle delivery to MSCs influences early mineralization of engineered cartilage templates *in vitro* and their subsequent ossification *in vivo*. Despite accelerating mineralization of engineered cartilage templates *in vitro*, RALA conjugated α -TCP nanoparticle delivery did not enhance endochondral bone formation *in vivo*. Therefore the potential of RALA conjugated α -TCP nanoparticle delivery appears to be as an alternative to growth factor delivery as a single stage strategy for promoting bone generation.

1. Introduction

Bone tissue engineering approaches commonly use temporary matrices in the form of scaffolds and hydrogels loaded with growth factors and/or exogenous mesenchymal stem cells (MSCs) to enhance regeneration¹⁻⁴. To date two distinct strategies have been employed to engineer functional bone using MSCs. The most commonly adopted approach is the direct conversion of MSCs in the engineered construct to bone forming cells, the deposition of bone specific matrix followed by its mineralization through simulating the process of intramembraneous ossification. This is commonly implemented by employing hydrogels or scaffolds loaded or stimulated with growth factors that induce osteogenic differentiation of the incorporated or recruited MSCs⁵⁻⁸. An alternative approach is the engineering of a hypertrophic cartilaginous template by inducing chondrogenic lineage differentiation in bone marrow derived MSCs in order to recapitulate the developmental process of endochondral ossification⁹⁻¹¹.

Several studies have convincingly demonstrated the potential of these approaches for functional bone regeneration, however a number of challenges remain. A well-documented concern relates to undesired side-effects associated with the supra-physiological delivery of growth factors *in vivo*^{12,13}. Alternatively, when MSCs or engineered tissues are delivered to a site of bone injury, the immediate low-oxygen environment which is typical at the implantation site can negatively affect their survival rate^{14,15}. Bone regeneration *via* an engineered cartilage template (endochondral tissue engineering) can theoretically address the challenges associated with growth factor delivery and the survival of cells as chondrocytes are better equipped to remain viable within such low-oxygen conditions. In addition, hypertrophic chondrocytes are known to produce pro-angiogenic factors which is advantageous for vascularization of the construct¹⁶⁻¹⁸. However, matrix mineralization using this approach largely depends on initiating a specific sequence of temporal molecular and cellular events starting from hypertrophy of chondrocytes, invasion of blood vessels, migration of osteoprogenitors and matrix mineralization¹⁶. The need for relatively longer *in vitro* culture periods for chondrogenic priming is also regarded as a hurdle that needs to be overcome to enable clinical translation of such approaches. Therefore investigating alternative strategies that facilitate control over stem cell lineage differentiation for functional bone formation (whether *via* intramembraneous or endochondral pathways) is critical to developing more translational approaches to bone tissue engineering.

The use of osteoinductive biomaterials to control stem cell fate has been well documented in the literature¹⁹. Calcium phosphate (CaP) based materials such as

hydroxyapatite (HA) and α - or β -tricalcium phosphate (α -TCP, β -TCP) have been extensively investigated as scaffolding materials for bone regeneration and many of them are in clinical use²⁰⁻²⁴. They are generally considered as osteoconductive and osteointegrative materials with good mechanical properties. However, recent literature has also demonstrated the osteoinductive properties of CaP based scaffolds in addition to their osteoconductive and osteointegrative properties^{19, 24-27}. Calcium ions and inorganic phosphate have been identified as key factors responsible for CaP based osteogenic differentiation and matrix mineralization²⁸⁻³⁰. Specifically, it has been demonstrated that bioactive factors such as BMP-2 are upregulated in stem cells in the presence of calcium ions^{29, 31}. Likewise, the role of CaP containing matrix vesicles in the mineralization of cartilage, bone and dentin has been clearly demonstrated in the literature³². Moreover their role as carriers of growth factors is also well documented^{33, 34}.

Direct delivery of CaP nanoparticle into the cytosol of stem cells represents a promising approach for the controlled release of calcium and phosphate ions and the formation CaP containing matrix vesicles (MVs). Even though CaP nanoparticles have shown their potential to enter the cytosol through endocytosis, problems associated with agglomeration of bare CaP nanoparticles and the hydrophilic nature of the particles can be limiting factors associated with their intracellular delivery. Therefore, delivery of CaP nanoparticles using a vehicle that can effectively pass the cell membrane and into the cytosol could significantly improve the efficiency of this approach. Recently, a 30-amino acid cell penetrating amphipathic peptide RALA, made up of repeating arginine/alanine/leucine/alanine units³⁵ has been shown to significantly improve the bioavailability of hydrophilic therapeutics³⁶⁻³⁸. The amphipathic RALA peptide interacts with the lipid-bilayers to transport cargo across cell membranes. The peptide can undergo endosomal disruption and escape due to the pH sensitive α -helicity of the peptide to facilitate delivery of its cargo to the cytosol without degradation. Moreover, RALA mediated TCP nanoparticle delivery has been recently shown to enhance matrix mineralization of MSCs in 2D culture in comparison to bare TCP particles³⁹. Based on these observations, we hypothesized that intracellular delivery of RALA conjugated α -TCP nanoparticle can trigger Ca^{2+} mediated osteogenic differentiation of MSCs and mineralization of the ECM to promote bone formation *in vivo*. Therefore, the overall objective of the study was to investigate the effect of RALA conjugated α -TCP nanoparticle delivery to MSCs embedded within alginate hydrogels on osteogenesis *in vitro* and bone formation *in vivo*. The therapeutic potential of α -TCP delivery will be evaluated within two different contexts. This study will

first evaluate RALA conjugated α -TCP nanoparticle delivery to MSCs as an alternative to growth factor delivery for promoting bone formation *in vivo*. Secondly, recognizing the potential and limitations associated with endochondral bone tissue engineering strategies, this study will then explore if α -TCP nanoparticle delivery to MSCs accelerates both mineralization of engineered cartilage templates *in vitro* and their subsequent ossification *in vivo*.

2. Materials and methods

2.1 Experimental design

This study was designed to investigate the osteogenic effect of delivering α -TCP nanoparticles conjugated to a RALA delivery peptide (hereafter known as *RTCP*) to MSCs. MSCs were either encapsulated in empty or growth factor (GF) loaded cell adhesion motif arginyl-glycyl-aspartic acid (RGD)⁴⁰ conjugated alginate hydrogels. Three systematic *in vitro* studies and two *in vivo* studies were performed. The composition of the hydrogels used in this study is summarized in Table 1. As part of *in vitro* study 1, osteogenic gene expression in MSCs following RALA mediated α -TCP delivery (hereafter known as *RTCP-MSC*) was evaluated over a 2 week culture period in the absence of external osteogenic growth factors. As part of *in vitro* study 2, the inherent bone forming potential of *RTCP-MSCs* was evaluated by encapsulating them in RGD-alginate hydrogels (hereafter known as *RTCP-MSC-hydrogel*). Constructs were cultured in the absence of osteogenic supplements and growth factors (GFs) for a period of 4 weeks. As part of *in vitro* study 3, the effect of RALA mediated α -TCP delivery to MSCs was evaluated in the presence of osteogenic and chondrogenic culture supplements. Untreated (control) MSCs (hereafter known as *C-MSC*) were encapsulated in RGD-alginate (hereafter known as *C-MSC-hydrogel*) as a control. For the *in vivo* studies, *MSCs* were encapsulated in RGD-alginate hydrogel loaded with the growth factors (BMP-2 and TGF β -3) which have previously been shown to promote bone formation following subcutaneous implantation into mice⁶ (hereafter known as *C-MSC-GF-hydrogel*). The synergistic effect of *RTCP* and *GF* delivery was also investigated in this study by evaluating *RTCP-MSCs* encapsulated in these *GF* delivery hydrogels (*RTCP-MSC-GF-hydrogel*). *C-MSCs-hydrogels* were used as controls. As part of *in vivo* study 1, bone formation following subcutaneous implantation of *RTCP-MSCs-hydrogel* and *RTCP-MSC-GF-hydrogel* was evaluated for a period of 8 weeks. Additionally, the synergistic effects of *RTCP* delivery and *GF*-delivery were evaluated. In the second *in vivo* study, the effect of 6 weeks of *in vitro* chondrogenic priming of *RTCP-MSC-hydrogels* and *C-MSC-hydrogel* on subsequent bone formation *in vivo* was

assessed. Experimental groups, conditions and time course of experiments are summarized in Table 2.

2.2 *RTCP nanoparticle formulation*

The synthesis of α -TCP and formulation of *RTCP* nanoparticles were carried out as we previously reported ⁴¹ Briefly, α -TCP ($\text{Ca}_3(\text{PO})_4$) nanoparticles were prepared using dicalcium phosphate (2CaHPO_4) and calcium carbonate (CaCO_3) precursors according to the solid state reaction given in Equation 1 ⁴²

Prepared nanoparticles were conjugated with RALA peptide by adding RALA (Biomatik, USA) and α -TCP in a ratio of 5:1, in ultra-pure water. Nanoparticles were allowed to form by spontaneous electrostatic interactions between the cationic peptide and anionic CaP at room temperature for approximately 30 min. Prepared *RTCP* nanoparticles were stored at 4°C for a period of 2-4 weeks before usage.

2.3 *MSC culture and intracellular delivery of RTCP.*

Bone marrow derived porcine MSCs were isolated according to a modified method developed for human MSCs ⁴³, expanded and cryopreserved as previously described ⁴⁴. Cryopreserved MSCs were expanded in T-175 flasks (Thermo Fisher Scientific, Ireland) at a density of 5000 cells per cm^2 in cell growth media (CGM) consisting of high glucose Dulbecco's modified eagle's medium GlutaMAX (hgDMEM), 10% (v/v) foetal bovine serum (FBS), 100 U mL^{-1} penicillin - 100 $\mu\text{g mL}^{-1}$ streptomycin (all from Gibco; Biosciences, Ireland) and 2.5 $\mu\text{g mL}^{-1}$ amphotericin B (Sigma-Aldrich, Ireland) Passage 2 MSCs were used for all studies. For intracellular delivery of *RTCP* nanoparticles, MSCs were plated at a density of 1×10^4 cells per cm^2 in T-175 cell culture flasks and incubated for 24 h in cell growth media. Prior to *RTCP* treatment, CGM was removed from the flasks and MSCs were washed twice using OptiMEM without serum (Biosciences, Ireland). OptiMEM containing *RTCP* (45.45 $\mu\text{g } \alpha\text{-TCP mL}^{-1}$) was prepared immediately before delivery procedure and was added to the cell culture flask. Control MSCs were prepared by treating them with OptiMEM containing an equal volume of ultrapure water. MSCs were incubated with nanoparticles at 37 °C with 5% CO_2 . At 6 h of incubation, media with *RTCP* was removed and washed once with CGM and incubated with fresh CGM overnight at 37 °C with 5% CO_2 . After overnight incubation, media was removed, washed 3 times with PBS and MSCs were trypsinized for further experiments.

2.4 Gene expression analysis

RTCP delivered MSCs and control MSCs were plated at a density of 5×10^5 cells per well in a 6-well plate and were incubated in cell neutral media (CNM) composed of hgDMEM supplemented with 3% FBS, 100 U mL^{-1} penicillin- $100 \mu\text{g mL}^{-1}$ streptomycin and $2.5 \mu\text{g mL}^{-1}$ amphotericin B for a period of 2 weeks at 37°C with 5% O_2 . Cell lysate for RNA isolation was prepared at 7 and 14 days of culture by lysing cells ($n=3$ wells/group/time point) using RLT lysis buffer (Qiagen, UK) supplemented with $10 \mu\text{L mL}^{-1}$ β -mercaptoethanol (Sigma-Aldrich, Ireland) and stored at -80°C . At the time of isolation, lysates were thawed and homogenized using a QIAshredder column (Qiagen, UK) and total RNA was isolated and purified using the RNeasy mini kit (Qiagen, UK) using the manufacturer's suggested protocol. Purity and yield of RNA was quantified using a NanoDrop Spectrophotometer (Labtech International, Uckfield, UK). For cDNA preparation, 50 ng total RNA per sample were reverse transcribed into cDNA per 20 μL of reaction volumes using a high capacity reverse transcription cDNA kit (Applied Biosystems, UK) as per manufacturer's instructions. Quantitative PCR was performed in an ABI 7500 sequence detection system (Applied Biosystems, UK) using SYBR select master mix (Applied Biosystems, UK) for evaluating the expression of Collagen type-1 (Col-1), Runt related transcription factor 2 (Runx-2), Bone morphogenetic protein 2 (BMP-2), Transforming growth factor beta 3 (TGF- β 3), Alkaline phosphatase (ALPL), Osteocalcin (BGLAP) and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes. Porcine specific primer sequences KiCqStart® SYBR® Green Primers; Sigma, Ireland) that were used for amplification of these genes are listed in Table 3. Comparative Threshold (cT) data were analysed using the $\Delta\Delta\text{CT}$ method as described previously⁴⁵ with GAPDH as the endogenous control. Relative expression of the genes is presented as fold changes relative to the control group.

2.5 Preparation RGD- γ alginate and encapsulation of MSCs

Gamma-irradiated (5 Mrad) RGD modified alginate (hereafter known as RGD- γ -alginate) was prepared as previously reported⁴⁶. Briefly, RGD peptides were covalently conjugated to γ - irradiated alginate using aqueous carbodiimide chemistry followed by purification and sterilization as previously described^{47, 48}. A 4% solution of RGD alginate was prepared in ultrapure water by stirring overnight in a 50 mL falcon tube aseptically. *RTCP-MSC* and *control MSCs* were prepared as mentioned above (2.3) and were suspended at a cell density of $20 \times 10^6 \text{ MSCs mL}^{-1}$ within RGD- γ -alginate (2%). To cast cylindrical hydrogels (5 mm diameter x 3 mm height) MSC-RGD- γ -alginate hydrogel solution was pipetted into sterile

agarose (3% final) molds with a final concentration 50 mM CaCl_2 and allowed to crosslink for 30 min at 37°C.

2.6 *In vitro* culturing of tissue engineered hydrogels

MSCs encapsulated hydrogels were cultured in osteogenic, chondrogenic or neutral conditions for various studies *in vitro*. For osteogenic conditions, the hydrogel-cell constructs were maintained in CGM supplemented with 100 nM dexamethasone, 10 mM β -glycerol phosphate, and 0.05 mM ascorbic acid (all from Sigma-Aldrich, Ireland) at 20% O_2 for 3 weeks. For chondrogenic conditions, tissue engineered hydrogels were maintained in CGM without FBS, supplemented with 100 $\mu\text{g mL}^{-1}$ sodium pyruvate, 40 $\mu\text{g mL}^{-1}$ L-proline, 50 $\mu\text{g mL}^{-1}$ L-ascorbic acid-2-phosphate, 1.5 mg mL^{-1} bovine serum albumin, 1X insulin-transferrin-selenium, 100 nM dexamethasone (all from Sigma-Aldrich, Ireland) and 10 ng mL^{-1} recombinant human TGF- β 3 (ProSpec-Tany TechnoGene Ltd., Israel) for 3- 6 weeks at 5% O_2 . For evaluating the intrinsic capability of *RTCP-MSCs* to form bone, and to evaluate the bone formation potential of GF delivery hydrogel *in vitro*, the hydrogel-cell constructs were maintained in CNM prepared as mentioned above (2.5) for 4 weeks. In all culture conditions, media change was performed twice weekly.

2.7 Cell viability assessment using live/dead staining

Live/dead staining at predetermined time points were performed using LIVE/DEAD™ viability/cytotoxicity assay kit (Invitrogen, Bioscience, Ireland) to evaluate the viability of MSCs in *RTCP-MSC-hydrogel* compared to that of *C-MSC-hydrogel*. Briefly, hydrogels were washed in PBS, sliced into halves and were incubated in 2 μM calcein AM and 4 μM ethidium homodimer-1-containing phenol-free DMEM (Sigma-Aldrich, Ireland). After 1 hour incubation, hydrogel-cell constructs were washed again in PBS and viability of encapsulated MSCs were assessed using a scanning confocal microscope (Olympus FV-1000, UK) at an excitation and emission wavelengths of 515 and 615 nm. Analysis was performed in FV10-ASW 2.0 Viewer software.

2.8 *In vivo* subcutaneous implantation

All animal experiments were approved by the ethics committee of Trinity College Dublin and the Irish Medicines Board (IMB). Six different groups (Table 2) were subcutaneously implanted subcutaneously into the back of transgenic Balb/C nude mice (Harlan, UK) ($n = 8$) using a previously described surgical procedure⁴⁹. For implantation, two subcutaneous pockets (one in the shoulder level and one in the hip level) were created per animal under ascetic

conditions. Next, three hydrogel-cell constructs were implanted per pocket (n= 6 per mouse). All the constructs were harvested at 8 weeks of *in vivo* implantation. Bone formation was analysed based on the gross appearance, micro computed tomography (μ CT) scan, biochemical evaluation and histology.

2.9 μ CT analysis

μ CT scans were performed soon after implant retrieval. All samples were scanned with a high resolution μ CT imaging system (Scanco Medical, Bassersdorf, Switzerland) at a resolution of 10 μ m, a voltage of 100 kV, and current of 100 mA. Volumetric reconstructions and analysis of the acquired data were performed by NRecon and CT Analyzer software provided by SkyScan. A cylindrical volume centred over the defect covering the entire construct was selected as the volume of interest (VOI). A global threshold of 70–255 was used for analysing the constructs. Reconstructed 3D images generated from the scans were used to visualize mineral distribution throughout the constructs.

2.10 Biochemical analysis for sulphated glycosaminoglycan (sGAG), collagen and calcium content.

Hydrogel-cell constructs were digested with papain enzyme as previously reported⁵⁰. Briefly, papain buffer extract (PBE) was prepared using 100 mM sodium phosphate buffer containing 0.1 M sodium acetate, 0.05 M ethylenediaminetetra-acetic acid, pH 6.0 (all from Sigma-Aldrich, Ireland). Papain (125 μ g mL⁻¹, Sigma-Aldrich, Ireland) was activated with 10 mM cysteine hydrochloride immediately before usage. DNA content was quantified using Hoechst Bisbenzimidazole 33258 dye assay (Quant-iT ssDNA Assay Kit, Biosciences). sGAG levels in the samples were measured using dimethylmethylene blue dye-binding assay, pH 1.35 (Blyscan, Biocolor Ltd., UK). For the collagen assay, papain digested samples were hydrolysed by treating with concentrated HCl (37%) and collagen content in the samples was quantified by measuring their hydroxyproline content using the Chloramine-T assay as previously described⁵¹. Acid digested hydrogel samples were used for the calcium assay. Briefly, hydrogel-cell constructs were digested with 1 M HCl and calcium in the samples was measured using standard calcium assay kit (Sentinel Diagnostics, Italy). Collagen and sGAG content in the constructs were normalised with DNA content and the calcium amount was normalised with the wet weight of the samples.

2.11 Histological evaluation of the constructs

Hydrogel-cell constructs were fixed in 4% paraformaldehyde (Sigma-Aldrich, Ireland) at the end of study periods, dehydrated and wax embedded in paraffin. Seven- μ m thick sections were sliced from the paraffin embedded samples using a microtome (Leica, Germany). Sections were stained with aldehyde fuchsin/alcian blue (AF/AB) for visualizing sGAG, standard picosirius red (PSR) for evaluating the collagen deposition, and alizarin red for evaluating the calcium deposition as previously described ⁹ . Semi-quantitative analysis of the stained area was performed using ImageJ (National Institute of Health).

2.12 Statistical analysis

Statistical analyses were performed by GraphPad Prism software (v.6, GraphPad, USA). Quantitative findings are presented as mean $\pm$ SEM. Student's t-test was used to compare differences between two experimental groups. Multifactor analysis of variance (ANOVA) was performed followed by Tukey's multiple comparison test to determine statistical significance when comparison was made using more than two groups. The difference between groups was considered statically significant at $p < 0.05$.

3. Results

3.1. RALA conjugated α -TCP nanoparticle delivery results in upregulation of osteogenic genes in MSCs

Delivery of *RTCP* to MSCs in monolayer *in vitro* culture induced upregulation of osteogenic genes (Fig. 2). Growth factor related genes such as BMP-2 and TGF- β 3 showed significant upregulation in *RTCP* treated groups compared to controls at 1 week. The expression of these genes returned to levels similar to that of untreated controls after 2 weeks. The osteogenic transcription factor Runx-2 was found to be upregulated at week 2. The osteogenic marker genes collagen type 1 and osteocalcin were also found to be significantly upregulated after two weeks in *RTCP* treated MSCs compared to controls.

3.2 RALA conjugated α -TCP nanoparticle delivery to MSCs encapsulated in alginate hydrogels accelerates mineralization *in vitro*

RTCP-MSC remained viable over 4 weeks *in vitro* following encapsulation in alginate hydrogels, demonstrating comparable levels of viability to *C*-MSC (all groups maintained in media containing 3% serum with no other growth factors added to the media) (Fig.3 A). H&E staining revealed similar levels of tissue deposition in both groups in CNM (Fig.3 B). However, more intense alizarin red staining was observed in the *RTCP*-MSC-hydrogels. In agreement

with histological findings, biochemical quantification confirmed significant increases in calcium deposition in the *RTCP-MSH-hydrogels* in comparison to *C-MSH-hydrogels* (Fig.3C).

3.3 RALA conjugated α -TCP nanoparticle delivery to MSCs supports mineralization in both chondrogenic and osteogenic conditions

RTCP-MSHs remained viable in alginate hydrogels incubated in both chondrogenic and osteogenic culture conditions (Fig.4). There was no difference between *RTCP-MSH-hydrogels* and *C-MSH-hydrogels* in terms of cell viability after 1 week in chondrogenic media or osteogenic media. Cells were found to be more aggregated in *RTCP-MSH-hydrogels* in comparison to *C-MSH-hydrogels* in chondrogenic culturing conditions (Fig.4 A). *RTCP-MSH-hydrogels* appeared to contain a higher number of viable cells compared to controls when maintained in osteogenic media (Fig.4 B). In agreement with this, the DNA content of *RTCP-MSH hydrogels* was higher in osteogenic conditions (Fig.4C), while no significant differences in DNA content were observed in chondrogenic conditions (Fig.4D).

RTCP-MSH maintained their capacity to undergo chondrogenic and osteogenic differentiation following encapsulation in alginate hydrogels (Fig.5). In the presence of chondrogenic differentiation factors, *RTCP-MSH-hydrogels* contained similar levels of sGAGs and collagen to *C-MSH-hydrogels* (Fig.5 A). Nodules of mineral were observed in *RTCP-MSH-hydrogels* maintained in chondrogenic conditions which were absent in *C-MSH-hydrogels* (Fig.5 A). In the presence of osteogenic differentiation factors, staining for sGAG accumulation was more intense in *RTCP-MSH-hydrogels*, with no apparent differences in collagen accumulation as evidenced by picosirius red staining. Staining for mineralization was more peripheral in *C-MSHs-hydrogels* but more homogenous in *RTCP-MSHs-hydrogels* (Fig.5 B).

3.4 The in vivo bone forming potential of RALA conjugated α -TCP nanoparticle delivery to MSCs is comparable to growth factor delivery

After 8 weeks *in vivo*, *RTCP-MSH-hydrogels*, *C-MSH-GF-hydrogels* and *RTCP-MSH-GF-hydrogels* had all formed a mineralized, bone-like tissue, while little evidence of calcification was observed in *C-MSH-hydrogels* (Fig.6. A and C). This was confirmed by microCT scanning, which revealed no statistically significant difference in mineral volume between the *RTCP-MSH-hydrogel*, *C-MSH-GF-hydrogel* and *RTCP-MSH-GF-hydrogel* groups. The *RTCP-MSH-hydrogels* contained significantly higher levels of mineral compared to the *C-MSH-hydrogels* (Fig.6 G). The combination of *RTCP* delivery and growth factor delivery in RGD-alginate hydrogel did not lead to a synergistic increase in mineral deposition. Histological analysis

showed a similar pattern of results when compared to the μ CT findings. There was no evidence of bone-like tissue formation (haematoxylin/eosin staining) or mineralization (alizarin red staining) in *C-MSC-hydrogels* compared to the other three groups (Fig.6 B and D). There was no statistically significant difference in collagen content among any of the groups (Fig.6E). Bone volume (quantified from histological images) was significantly higher following either RTCP delivery or growth factor delivery (Fig.6 F).

3.5 Despite accelerating mineralization of engineering cartilage templates in vitro, RALA conjugated α -TCP nanoparticle delivery does not enhance endochondral bone formation in vivo

RTCP-MSC-hydrogels and *C-MSC-hydrogels* constructs were cultured for 6 weeks in chondrogenic growth conditions *in vitro* to engineer a cartilage template. Histological analysis revealed similar levels of sGAGs and collagen in both groups (Fig.7 A), however *RTCP-MSC-hydrogels* contained higher levels of calcium (Fig.7A, B). Bone formation in all chondrogenically primed constructs showed similar levels of bone formation 8 weeks after subcutaneous implantation (Fig.7 C). There were no significant differences in collagen content and mineral volume between the groups (Fig.7 D).

4. Discussion

The goal of this study was to investigate the potential of RTCP delivery to MSCs as a promising translational approach to generate engineered bone tissue. α -TCP nanoparticles conjugated to a RALA delivery peptide were prepared and delivered to MSCs prior to encapsulation into an RGD-modified alginate hydrogel. The results from this study support the hypothesis that delivering RTCP to MSCs accelerates mineralization *in vitro* and improves bone formation *in vivo*. Of note was the finding that the osteoinductive effect of RTCP delivery was similar to that of delivering a combination of growth factors (BMP-2 and TGF- β 3) that was previously shown optimal for promoting bone formation⁶. In addition, RTCP delivery showed its potential to facilitate enhanced mineralization of engineered cartilage templates for endochondral bone formation strategies. Taken together, the results from this study demonstrate that a combinatorial approach involving MSCs and RTCP delivery has the potential to be used as a single stage strategy for bone regeneration without the need for exogenous growth factors.

Conjugation of α -TCP with a RALA peptide has previously been shown to improve the intracellular delivery of CaP nanoparticles^{39, 41, 52}. Conjugation of the α -TCP nanoparticles to the RALA peptide limits agglomeration, with the suspension being stable for a period of 4 weeks at 4-37°C⁴¹. In addition, the presence of the cell penetrating peptide RALA acts to

mitigate the hydrophilicity-associated negative effects linked to bare CaP nanoparticles²⁸ in terms of their capability to enter the cell membrane. CaP based biomaterials, including α and β -TCP, hydroxyapatite (HA) and calcium sulphate, have been extensively used in orthopaedics as a bone substitute material^{26,53,54}. Nanoparticles in a size range of 20-200 nm has been shown to be optimal for intracellular delivery, with bare CaP nanoparticles often undergoing significant levels of agglomeration leading to development of a much larger particle size over time⁵⁵⁻⁵⁸. Thus, currently CaP nanoparticles are largely used with modifications to increase their stability and to control their size in order to improve their capacity to cross the cell membrane⁵⁷. Even though use of CaP based nanoparticles as carriers for intracellular delivery of nucleic acids has been explored for 40 years, the inherent osteogenic effect of calcium and phosphate ions released from such particles has not been considered in the majority of the studies. This is despite the fact that several studies have separately reported the osteoinductive properties of CaP based agglomerates and bulk scaffolding materials^{26,27,59}.

RTCP delivery to MSCs lead to an upregulation of the master osteogenic transcription factor Runx-2 and other osteogenic genes (Fig. 3) which was preceded by an increase in BMP-2 expression. The potential of Ca²⁺ ions as osteoinductive cues is well established²⁹⁻³¹. For example, a three-fold upregulation of BMP-2 in human bone marrow derived MSCs has previously been reported in response to high extracellular Ca²⁺ concentration²⁹. Activation of protein kinase K (PKA) followed by Ras-MAP-kinase signalling pathway by Ca²⁺ ions which are internalized, possibly through type-L voltage-gated calcium channels, has been proposed as the possible mechanism of BMP-2 activation²⁹. This confirms that BMP signalling is one of the key events in RTCP mediated osteogenesis in MSCs. In the case of intracellular delivery of CaP nanoparticles using RALA mediated delivery, Ca²⁺ ions can be readily available in the cytosol for activating PKA. A similar effect was observed after MSCs were encapsulated into alginate hydrogels, where α -TCP nanoparticle delivery also lead to increases in construct mineralization *in vitro*.

In addition to enhancing osteogenesis of MSC *in vitro*, RTCP delivery to MSCs induced similar levels of bone formation *in vivo* to that of growth factor delivery from hydrogels. Incorporation of RGD to alginate has been previously shown to create attachment points to control phenotype, enhance cell-matrix interactions and promote differentiation into mature osteoblasts from mouse and human pre-osteoblasts^{60,61} and human MSCs⁶². In addition, delivery of BMP-2 from these low molecular weight hydrogels have been shown to robust bone regeneration *in vivo*^{6,63}. BMP-2 is long established as having potent osteoinductive properties

^{64, 65}, however there are well documented adverse outcomes associated with their use ⁶⁶⁻⁶⁸. A recent review on the complications associated with BMP-2 delivery identified unanswered questions related to optimal dosing, repeat exposures, carcinogenesis and long-term results ¹². The fact RTCP delivered to MSCs encapsulated within alginate hydrogels showed similar level of bone formation to that of growth factor delivery from hydrogels points to their use as an alternative to BMP-2 delivery for the repair of large bone defects. Further evaluation of these constructs in orthotopic defects is required to validate these proof-of-concept findings.

RTCP delivery to MSCs accelerated mineralization of the matrix within tissue engineered constructs maintained in non-specific culture conditions and those known to promote chondrogenesis and endochondral ossification. During bone formation, osteoblast produce bone specific extracellular matrix which is mineralized with HA nanocrystals ⁶⁹. The unique organization of mineralized collagen matrix provides structural integrity and mechanical strength to bone. The role of CaP-containing vesicles in the matrix mineralization has been previously established ^{28, 32-34, 70}. This suggested that the release of Ca^{2+} and PO_4^{3-} ions from RTCP nanoparticles in the cytosol of MSCs enhanced Ca^{2+} and PO_4^{3-} accumulation in the MVs, resulting in enhanced mineralization of the matrix. However, delivery of RTCPs does not seem to have interfered with the chondrogenic potential of MSCs. This is an important finding which can have important implications in endochondral bone regeneration strategies. For example, it might enable shortening of the *in vitro* priming time necessary to engineer hypertrophic cartilage templates suitable for bone regeneration. Of note is the finding that the mineral volume of bony tissues generated *in vivo* from engineered cartilage templates was noticeably higher than that generated by either growth factor delivery or α -TCP nanoparticle delivery (compare Fig. 6 and Fig. 7). Furthermore, these cartilaginous templates were noticeably more vascularized than other groups, suggesting that both nanoparticle and growth factor delivery strategies can be further improved to enhance their osteoinductivity.

In conclusion, the results of this study clearly demonstrate the osteo-inductive potential of RTCP nanoparticle delivery to MSCs for bone tissue engineering. Their delivery to MSCs results in increased expression of osteogenic growth factors and transcription factors, accelerated mineralization and robust bone development *in vivo*. Achieving osteogenic differentiation without the use of exogenous growth factors is a new and exciting approach in bone tissue engineering. This study demonstrates the potential of CaP nanoparticle delivery to

drive osteogenesis and hence act as such as alternative to traditional growth factor delivery approaches.

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Figure Legends

Fig.1: (A) Schematic representation of RALA-TCP (*RTCP*) nanoparticle preparation and delivery to MSC to obtain *RTCP* delivered MSC (*RTCP-MSC*). (B) Schematic representation of *RTCP-MSC* laden hydrogel preparation, and the hypothetical description of molecular events leading *RTCP* induced bone formation.

Fig.2: Expression of growth factor genes and osteogenic marker genes in RALA conjugated TCP treated MSCs related to untreated controls at week 1 and 2 of in vitro culture. * $P < 0.05$ in upregulation and + $P < 0.05$ in downregulation compared to untreated control ($n = 3$).

Fig.3: Long term viability, tissue formation and mineralization of RALA conjugated TCP treated MSCs (*RTCP-MSC*) and control MSCs (*C-MSC*) in alginate hydrogels in the absence of external growth factors. (A) is the live/dead confocal images of bare alginate hydrogels at 7 and 28 days of in vitro culture in neutral media. (B) is the histological images of bare alginate hydrogels cultured in neutral media for 28 days stained with H&E and alizarin red for detecting tissue formation and mineralization respectively. (C) is the biochemical quantification of calcium content in the bare alginate hydrogels incubated in cell neutral media (CNM) for 28 days ($n = 4$, * $P < 0.05$). Histology scale bar low magnification 500 μm , high magnification 100 μm .

Fig.4: Viability and proliferation of *RTCP-MSC* in alginate hydrogels. Representative live/dead images of *RTCP-MSC-hydrogels* and *C-MSC-hydrogels* at 1 week in (A) chondrogenic and (B) osteogenic media respectively. DNA amount in the hydrogels at 3 weeks in chondrogenic and osteogenic growth conditions are shown as (C) and (D) respectively. * $P < 0.05$ compared to *C-MSC-construct* ($n = 5$).

Fig.5: Effect of RALA conjugated TCP treated MSCs (*RTCP-MSC*) and control MSCs (*C-MSC*) laden hydrogels in chondrogenesis, osteogenesis and mineralization in osteogenic and chondrogenic culture conditions. (A and B) are the histological images of the tissue engineered

hydrogels incubated in chondrogenic media for 3 weeks stained with aldehyde fuchsin/alcian blue (AF/AB), picrosirius red and alizarin red for detecting the presence and distribution of sGAGs, collagen and mineralization respectively. Black arrow in (A) shows mineral deposits in the hydrogels even in chondrogenic conditions. Scale bar low magnification 500 μm , high magnification 100 μm .

Fig.6: Bone formation in the hydrogels 8 weeks after *in vivo* implantation. (A) Representative gross morphology (a, c) and μCT images (b, d) of control MSCs (C-MSC) and RALA conjugated TCP treated MSCs (RTCP-MSC) laden alginate hydrogels. (B) Representative images of C-MSC and RTCP-MSC laden alginate hydrogel sections stained with haematoxylin/eosin and alizarin red respectively. (C) Representative gross morphology (e, g) and μCT images (f, g) of C-MSC and RTCP-MSC laden growth factor (GF)-loaded alginate hydrogels. (D) Representative histological images of C-MSC and RTCP-MSC laden GF-loaded alginate hydrogel sections stained with haematoxylin/eosin and alizarin red respectively. (E) Biochemical quantification of collagen content in the hydrogels (n=3). (F) Bone tissue volume quantified from the histological images of the hydrogels (n=5, *P< 0.05). (G) Mineral volume quantified using μCT imaging (n=8, *P< 0.05). Histology scale bar low magnification 500 μm , high magnification 100 μm .

Fig.7: Bone formation in tissue engineered hydrogels first primed *in vitro* in chondrogenic media for 6 weeks before *in vivo* implantation for 8 weeks. (A) Representative images of control MSCs (C-MSC) and RALA conjugated TCP treated MSCs (RTCP-MSC) laden alginate hydrogel sections stained with aldehyde fuchsin/alcian blue (AF/AB) for sGAGs, picrosirius red for collagen and alizarin red for mineralization respectively after 6 weeks of *in vitro* chondrogenic priming. (B) Biochemical quantification of collagen (n = 5) and calcium (n = 5) content of engineered tissues after 6 weeks of *in vitro* priming. (C) Representative gross morphology (a and c), μCT image (b and d) of C-MSC and RTCP-MSC laden alginate

hydrogels, as well as sections stained with haematoxylin/eosin and alizarin red respectively 8 weeks after *in vivo* implantation. (D) Biochemical quantification of collagen content ($n = 3$) and μ CT quantification of mineral volume ($n = 8$) in the hydrogels 8 weeks after *in vivo* implantation. Histology scale bar low magnification 500 μm , high magnification 100 μm .

Fig.1

Fig.2

Fig.3

Fig.4

Fig.5

Fig. 6

Fig.7

Table-1: Composition of tissue engineered hydrogels

Constructs	RGD-alginate	Growth Factor	<i>Control-MSC</i>	<i>RTCP-MSC</i>
-	-	-	√	√
<i>C-MSC-hydrogel</i>	√	-	√	-
<i>RTCP-MSC- hydrogel</i>	√	-	-	√
<i>C-MSC-GF- hydrogel</i>	√	√	√	
<i>RTCP-MSC-GF- hydrogel</i>	√	√	-	√

Table 2: Experimental groups, conditions and duration of each study

	Studies	Experimental groups	Sample/Construct types	Cell neutral media	Osteogenic media	Chondrogenic media
In vitro	Study -1	Group-1	<i>C-MSC-monolayer</i>	1 and 2 weeks		
		Group-2	<i>RTCP-MSC-monolayer</i>	1 and 2 weeks		
	Study -2	Group-3	<i>C-MSC-hydrogel</i>		4 weeks	
		Group-4	<i>RTCP-MSC-hydrogel</i>		4 weeks	
	Study -3	Group-5	<i>C-MSC-hydrogel</i>			3 weeks
		Group-6	<i>RTCP-MSC-hydrogel</i>			3 weeks
			Construct types	In vitro chondrogenic priming period	In vivo period	Number of hydrogels
In vivo	Study -1	Group-1	<i>C-MSC-hydrogel</i>	None	8 weeks	8
		Group-2	<i>RTCP-MSC-hydrogel</i>	None	8 weeks	8
		Group-3	<i>C-MSC-GF-hydrogel</i>	None	8 weeks	8
		Group-4	<i>RTCP-MSC-GF-hydrogel</i>	None	8 weeks	8
	Study -2	Group-5	<i>C-MSC-hydrogel</i>	6 weeks	8 weeks	8
		Group-6	<i>RTCP-MSC-hydrogel</i>	6 weeks	8 weeks	8

Table 3. Primer sequences used for real-time PCR

Gene	Forward primer	Reverse primer
COL1A1	TAGACATGTTTCAGCTTTGTG	GTGGGATGTCTTCTTCTTG
RUNX2	CCAACAGAGGCATTTAAGG	CCAAAAGAAGTTTGCTGAC
BMP-2	ATGTGGAGGCTCTTCAATG	CATGGTCGACCTTTAGGAG
TGF β 3	GAGTACTATGCCAAGGAAATC	TTGAAGCGGAAAATCTTGG
ALPL	TTTCACTCTTCTTAGTGCTG	CGTTACGGAATGAGGAAAC
BGLAP	CAGGAGGGAGGTGTGTGAG	TGCGAGGTCTAGGCTATGC
GAPDH	TTTAACTCTGGCAAAGTGG	GAACATGTAGACCATGTAGTG