

Full length article

Localized growth factor delivery from microparticles modulates osteogenic and chondrogenic gene expression in a growth factor-dependent manner in an *ex vivo* chick embryonic bone model

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ABSTRACT

Growth factors play a crucial role in regulating various cellular functions, including proliferation and differentiation. Consequently, the biomaterial-based delivery of exogenous growth factors presents a promising strategy in regenerative medicine to manage the healing process and restore tissue function. For effective therapeutic applications, it is essential that these active compounds are precisely targeted to the site of regeneration, with release kinetics that align with the gradual pace of tissue growth. We have developed an *ex vivo* model utilizing a developing embryonic chick bone, and using PLGA-based microparticles as controlled-release systems, allowing for the investigation of the spatiotemporal effects of growth factor delivery on cell differentiation and tissue formation. Our findings demonstrate that BMP2 and FGF2 can significantly alter cell morphology and zonally pattern collagen deposition within the model, but only when the growth factor presentation rate is carefully regulated. Furthermore, the growth factor-dependent responses observed underscore the potential of this model to explore interactions between cells and the growth factors released from biomaterials in an approach which can be applied to bone tissue engineering.

Statement of significance: Current biomaterial-based strategies for bone tissue engineering face critical limitations in mimicking the spatial and temporal dynamics of native tissue development. This study introduces an innovative *ex vivo* embryonic chick bone model to evaluate localized, sustained growth factor delivery using PLGA microparticles. By precisely controlling the release of BMP2 and FGF2, the research demonstrates growth factor-specific modulation of osteogenic and chondrogenic gene expression and matrix deposition, outcomes that traditional *in vitro* models fail to capture. This physiologically relevant platform bridges a critical gap between basic *in vitro* assays and complex *in vivo* models, offering a powerful, low-cost tool for preclinical screening of regenerative therapies, and advancing the rational design of next-generation bone healing strategies.

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1. Introduction

Bone regenerative therapeutics focus on the restoration of osseous defects and in particular critical-sized defects resulting from revision surgery, osteonecrosis, pathological deformation, traumatic injuries, infection and tumor resection [1]. While bone grafting is regarded as the gold standard procedure of choice, limited donor site availability and donor site morbidity remain significant challenges of autologous bone grafting. Furthermore, rejection and infection are considered as major drawbacks of allogenic bone grafts [2]. This has heightened the demand for synthetic tissue-engineered alternatives to address complex clinical conditions, such as non-union fractures [3]. Scaffolds with desired mechanical and physical properties have been considered as alternative approaches to facilitate the regeneration process. However, the lack of functional moiety to promote interaction with human progenitor cells and insufficient biomimetic properties are major challenges that must be addressed to promote functional tissue formation using simple polymeric biomaterials [4]. To overcome these limitations, strategies that integrate cell and growth factor delivery with biomaterial scaffolds have shown promise in achieving superior regenerative outcomes [5–7].

To date, various growth factors have been explored for their potential applications in bone and cartilage tissue engineering, including bone morphogenetic protein 2 (BMP2) [8–10], bone morphogenetic protein 7 (BMP7) [11], transforming growth factor beta 1 (TGFβ1) [12,13], transforming growth factor beta 2 (TGFβ2) [14], transforming growth factor beta 3 (TGFβ3) [15], platelet-derived growth factor (PDGF-BB) [16], vascular endothelial growth factor (VEGF) [17], and fibroblast growth factor 2 (FGF2) [18] have been examined for bone tissue engineering. A substantial portion of research in this field has concentrated on the potential therapeutic application of BMP2 [19,20]. While significant improvement in healing has been reported following BMP2 delivery at bone defect or fracture sites, several adverse effects have been reported, including ectopic bone formation, osteolysis, retrograde ejaculation and increased risk of malignancy [21–23]. It has been speculated that these adverse effects may be linked to the delivery of supraphysiological concentrations of BMP2 due to insufficient or absent sustained release mechanisms [21,24].

To enhance efficacy while reducing dosages, the field has shifted toward the manufacturing of scaffolds incorporating a lower quantity of bioactive cargo with sustained release over time [25]. To achieve the desired therapeutic outcomes, various sophisticated carriers have been manufactured for bone tissue engineering to enable spatiotemporal delivery of single or multiple growth factors and recombinant morphogens [26]. The effect of a growth factor is governed by various factors including; i) the concentration of the growth factor, ii) the cell type and the receptors involved, and iii) the intracellular transduction pathways at play [27]. Despite recent advances in fabrication of growth factor-loaded materials, lack of appropriate biological *in vitro* assays to assess their release profile and efficacy has remained as one of the primary obstacles [28]. A number of approaches have been developed to test manufactured polymeric systems; including, *in vitro* osteoinduction assays [29–31], subcutaneously implanted diffusion chambers in SCID mice [32] and bone-on-a-chip microfluidic platforms [33]. However, these approaches lack the complexity to fully delineate the therapeutic potential of developed growth factor-loaded polymers in bone tissue engineering. Therefore, a more physiologically relevant model is highly desirable to accurately assess the efficacy and clinical utility of polymeric carriers containing single, dual or multiple growth factors.

Bone development in embryonic chicks follows a similar developmental pathway as human bone during endochondral ossification [34, 35] making embryonic chick bones a useful model for bone regeneration, as reviewed in Marshall et al., 2020 [36]. In this study, the organotypic embryonic chick bone was developed as a more physiologically relevant model to analyze the localized effect of BMP2 released from PLGA-based MPs (BMP2-MPs) with 10 % w/w PLGA-PEG-PLGA tri-block copolymer as plasticizer and average diameter of 20µm alongside

appropriate controls. Additionally, FGF2-loaded MPs (FGF2-MPs) were injected to evaluate cellular response to a non-osteoinductive growth factor, with the aim of determining growth factor dependency of cellular response in the chick embryonic hyaline cartilage.

2. Materials and methods

2.1. Microparticle manufacturing and growth factor encapsulation

Poly(vinyl alcohol) (PVA, molecular weight: 13000 – 23000 Da, 87–89 % hydrolyzed), human serum albumin (HSA, Sigma-Aldrich, UK), Poly (DL-lactide-co-glycolide) (PLGA) polymers with lactide: glycolide ratios of 50:50 (DLG 4.5A 59 kDa) were purchased from Evonik Industries (Alabama, USA). Recombinant human BMP2 (BMP2) was obtained from 2 different sources including Professor Walter Sebold (University of Wurzburg, Germany) and ORF Genetics (BMP2^{ORF}, ORF Genetics Ltd., Iceland). Recombinant human FGF2 was purchased from ORF Genetics (ORF Genetics Ltd., Iceland). PLGA-PEG-PLGA tri-block was produced in house following the protocol published previously [37]. PLGA-based MPs were manufactured using a water-in-oil-in-water (w/o/w) double emulsion method and characterized as previously described [37]. Briefly, PLGA-PEG-PLGA tri-block copolymer was added to PLGA to provide weight percentages of 10 % (w/w) of the 1g total mass in 5 ml dichloromethane. The total protein loading within the MPs was based on a formulation in which HSA comprised approximately 90 % (90 mg/ml) and BMP2 made up 10 % (10 mg/ml) of the total protein content, corresponding to an HSA:BMP2 ratio of 9:1. The polymer was used at a mass of 1 gram in 5 mL dichloromethane. At these concentrations a 100 µL aliquot of protein solution gave a protein loading of 1 % with respect to polymer mass [37]. These phases were homogenized for two minutes at 4,000 rpm in a Silverson L5M homogenizer (Silverson Machines, UK) to form the water-in-oil emulsion. This primary emulsion was transferred to 200 ml 0.3 % (w/v) polyvinyl alcohol (PVA) solution and was homogenized for a second time at 9,000 rpm. The resultant double emulsion was stirred at 300 rpm on a Variomag 15-way magnetic stirrer for a minimum of 4 hours to facilitate DCM evaporation, then washed and lyophilized (Edwards Modulyo, IMA Edwards, UK). The MPs were stored at -20 °C until injection. FGF2-MPs were manufactured following the same procedure. Fluorescently-labelled MPs were manufactured by addition of 0.001 % Coumatin-6 (Sigma-Aldrich, UK) as described previously [38].

2.2. Microparticle characterization

Microparticles were characterized as described elsewhere [37]. Briefly, a suspension of the microparticles was prepared in double deionized water and sized using a laser diffraction method to measure the size distribution of microparticles (Supplementary Fig. 1, Coulter LS230, fitted with the hazardous fluids module, Beckman Coulter, UK) while under agitation to prevent the particles settling. Microparticles of PLGA were gold sputter coated for 4.5 min at 30 mA (Baltzers SCD 030 gold sputter coater). Imaging was carried out with an accelerating voltage of 10 Kv (JEOL 6060LV variable pressure scanning electron microscope; Supplementary Fig. 2).

Encapsulation efficiency (EE) was determined based on total protein content, and the effective growth factor concentrations were predicted from this known ratio. In order to measure the encapsulation efficiency of protein within microparticles, 10 mg microparticles were incubated in 750 µl dimethyl sulfoxide (DMSO) at room temperature for 1 hour, followed by the addition of 150 µl of 0.5 % SLS/0.02 N NaOH for a further incubation at room temperature for another hour. Protein concentration of the resulting solution was measured using a bicinchoninic acid (BCA) protein assay kit (Thermo Scientific; USA) and compared against a standard curve of HSA conducted at the same time. 150 µl of sample were mixed with 150 µl of BCA reagent and incubated for 2 hours at 37 °C. Absorbance at 462 nm was measured using a Tecan Infinite 200

plate reader. To study the release profile, aliquots of the microparticles (100 mg) were suspended in 3 ml of phosphate-buffered saline (PBS) and then incubated on an orbital shaker set at 5 rpm at 37 °C. The PBS was completely replaced at regular intervals and the protein content was measured using the BCA protein assay kit (Supplementary Fig. 3). Calibration standards reflected the formulation of protein within the microparticles and were prepared in PBS.

2.3. Microinjection

In order to facilitate monitoring of the injection procedure under the microscope, GF-loaded MPs were mixed with labelled blank MPs in a ratio of 5:1. 100 mg of GF-loaded MPs and 20 mg of labelled blank MPs were resuspended in 1 ml PBS and were injected into various sites in the femur (both cartilaginous epiphyses and the mid-point of the diaphyseal bone collar) using a Femtojet system (Eppendorf, Germany) and glass capillary needles with a ~50–400 µm tip external diameter (Supplementary Fig. 4) pulled using a Flaming/Brown Micropipette Puller (Sutter, USA). 20 nl of suspension was injected into each site under sterile conditions with the aid of a dissecting microscope. Following optimization of the injection procedure, FGF2-MPs and BMP2-MPs were injected into chick embryonic bones. FGF2 solution (100 µg/ml), BMP2 solution (100 µg/ml) and other appropriate controls including 1 % HSA solution (carrier protein), and blank MPs (containing only HSA) were also injected following a similar procedure. Endotoxin free BMP2-loaded MPs (BMP2^{ORF}-MPs) were injected following a similar procedure to rule out the potential effect of endotoxin.

2.4. Organotypic culture of embryonic chick bones

Following injection, organotypic cultures of embryonic day 11 (E11) chick bones were performed as described previously [34]. Briefly, injected bones were placed onto Millicell inserts (0.4-µm pore size, 30-mm diameter; Millipore) in FalconTM six-well tissue culture plate (Becton Dickinson Labware, USA) containing 1 ml minimum essential medium alpha (α-MEM) (Gibco) containing 100 units penicillin, 100 µg/mL streptomycin, and 100 µM L-ascorbic acid 2-phosphate (Sigma-Aldrich) per well at the liquid/gas interface. Bones were cultured for 10 days at 37 °C, 5 % CO₂ with media changed every 24 h.

2.5. Histological examination

Bones were fixed in 4 % paraformaldehyde for at least 24h. Samples were processed through a series of graded ethanol, cleared in xylene and embedded in low-melting point paraffin wax. Ten micrometer sections were cut and stained with Alcian blue/Sirius red staining to assess proteoglycan and collagen contents as described previously [34]. Briefly, sections were first deparaffinized and rehydrated through graded alcohols and water. Then, sections were stained with Weigert's Hematoxylin and differentiated in acid/alcohol followed by staining with 0.5 % Alcian blue for proteoglycan-rich cartilage matrix and 1 % Sirius red for collagenous bone matrix. Subsequently, sections were dehydrated and cleared before mounting in DPX. Images were captured using the Nanozoomer Digital Pathology (Hamamatsu Photonics K.K., Japan).

2.6. Immunohistochemical (IHC) staining

Bone and cartilage markers were assessed by analyzing expression of collagen type I (COL-I), type II (COL-II), type X (COL-X) and osteocalcin (OC) as described previously [35]. Briefly, deparaffinized and rehydrated sections were treated in tri-sodium citrate buffer (Fisher, UK) for antigen retrieval. Then samples were washed in running water and incubated with 3 % hydrogen peroxide to quench endogenous peroxidase activity. Sections were digested with hyaluronidase for COL-II immunostaining. Non-specific binding was blocked with 1 % bovine serum albumin (BSA) followed by overnight incubation at 4 °C with primary antibodies diluted appropriately in 1 % BSA. Sections were subsequently washed in PBST (PBS + 0.5 % Tween 20) and incubated with biotinylated horse anti-rabbit IgG secondary antibody for COL-I and COL-II and biotinylated horse anti-mouse IgG secondary antibody (Vector Laboratories Inc) for OC diluted in 1 % BSA. ExtrAvidin[®] Peroxidase and 3-amino-9-ethylcarbazole (AEC) substrate solution (Sigma Aldrich) was used to visualize brown immune complex reaction product, with Alcian blue counter stain. Negative controls, either exclusion of only primary antibody or both primary and secondary antibodies, showed absence of any positive staining.

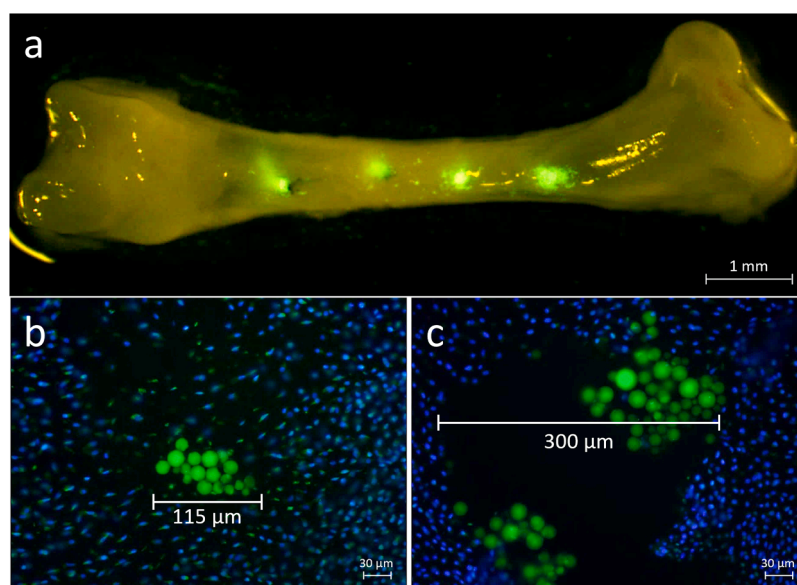


Fig. 1. MPs injected in both diaphyseal and epiphyseal regions (a). Minimal invasive damage achieved following microinjection of MPs using glass needle with long and narrow tips with external diameters of ~100 µm (b). Extensive damage recorded following microinjection using glass needle with shorter tips and wider orifice (c). Nucleus counterstained with 4', 6-diamidino-2-phenylindole (DAPI).

2.7. Environmental scanning electron microscopy

Ten micrometer sections were cut and mounted on Thermanox™ plastic coverslips (Nunc, USA). Then sections were deparaffinized and rehydrated through graded alcohols and water. Images were obtained using an environmental scanning electron microscope (Philips FEG ESEM) equipped with a Trecor detector set at an accelerating voltage of 10 kV under a vacuum of 4.0–5.8 Torr and 6–10 % humidity.

2.8. Image analysis

Images were captured using the Nanozoomer Digital Pathology (Hamamatsu Photonics K.K., Japan). The intensity was assessed by three-level thresholding of the COL-I staining of 6 independent samples using Image J software and a purpose-built macro (<https://github.com/DaleMoulding/Fiji-Macros?tab=readme-ov-file#3-level-dab-q-quantifier>).

Image analysis was performed on sections obtained from independent microinjections and sections from the same injections. In case of Blank-MP injected samples, image analysis was performed on four independent injections and at least two sections from each injection (total n=21). Analysis on BMP2-MPs injected to PZ was performed on four independent injections and two sections of one injection (PZ1 and 2) and one section of three other injections (PZ 3, 4 and 5; total n=5). For BMP2-MPs injected to CZ, analysis was performed on five independent injections and two sections of three samples (CZ1 and 2, CZ4 and 5 and CZ7 and 8) and one section of two other injections (CZ 3 and CZ6; total n=8).

2.9. Statistical analysis

Data were collected from at least three biological replicates and analyzed by GraphPad Prism (version 10). We did not use statistical methods to predetermine sample size, there was no randomization designed in the experiments, and the studies were not blinded. Significance was determined by One-way analysis of ANOVA with Tukey's multiple comparisons test with a single variance. Data are represented as mean $\pm$ SD, * p < 0.05, ** p < 0.01, *** p < 0.001. **** p < 0.0001.

3. Results

3.1. Injection procedure

To achieve a minimally invasive injection, a range of glass needles with external diameters of 50–400 μ m were initially tested (Supplementary Fig. 4). Following injection, embryonic chick bones injected with fluorescently-labelled MPs were sectioned and imaged. As minimal-invasive injection and retention of injected MPs in close proximity to host cells was of utmost importance to accurately evaluate the effects of released GFs, samples were examined microscopically for evidence of damage to tissue architecture surrounding injection sites and cell localization around the injected MPs. The results indicated that minimal damage with negligible alteration to the architecture of native tissue could be achieved using needles with defined narrow tips and an external diameter of $\sim$ 100 μ m. Measurement of three individually pulled needles indicated that needles with 103.3 ± 4.4 μ m in external diameter have an internal diameter of 35.1 ± 1.9 μ m. Although the volume and the number of injected MPs were increased 3–4 fold with the use of glass needles with wider bores, injection with such needles resulted in substantial tissue damage and the formation of large cavities (Fig. 1).

3.2. BMP2-MPs injection

To assess the cellular response to the delivered growth factors following injection, Alcian blue/Sirius red staining was performed on

injected samples. To ensure specificity of response, various control groups were included in the study including injection of 1 % HSA solution (carrier protein), 100 μ g/ml BMP2 solution, and blank PLGA microparticles containing 1 % HSA. Given that 20 nL of protein solution or resuspended BMP2-loaded microparticles (BMP2-MPs) were injected, the total delivered protein per injection site was approximately 2 ng. Based on a loading efficiency of 90.7 ± 16.4 % for the BMP2-MPs, the actual amount of BMP2 delivered via microparticles was 1.81 ± 0.33 ng per injection site. Microscopical analysis of sections of injected samples showed that morphological changes were limited to reformation of tissue surrounding the injection sites in control samples with no obvious cellular response. However, distinct morphological changes were observed in cells located in close proximity to the BMP2-MPs injection sites in the epiphyseal proliferative zone (PZ). Injection of BMP2-MPs into a zone containing pre-hypertrophic and hypertrophic zones (committed zone - CZ) demonstrated deposition of collagen (collagen fibers evidenced by Sirius red staining) rather than morphological changes (Fig. 2).

To further analyze the morphology of cells, BMP2-MPs injected samples were analyzed using ESEM. While native cells in the PZ of non-injected samples remained arranged in a dispersed fashion and exhibited the typical round morphology with no distinguishable cytoplasmic projections, cells in close proximity of injected BMP2-MP demonstrated several cytoplasmic projections within a compact arrangement (Fig. 3).

To examine the expression of osteogenic and chondrogenic markers, IHC analyses of samples were performed using antibodies against COL-I, COL-II, COL-X and OC. Since each of these osteogenic and chondrogenic markers are regulated differentially within the PZ and CZ (based on the level of progenitor commitment), a pronounced difference in level of expression in comparison with neighboring cells was considered as a positive response in injected or control groups. Expression of both COL-I and COL-II was noted to be up-regulated in PZ cells located in close proximity to injected BMP2-MPs in comparison to uncommitted native counterparts. In contrast, injection of BMP2-MPs into the CZ led to a more pronounced expression of COL-I (Fig. 4). Similarly, up-regulation of COL-I expression was also observed in cells in close proximity to injected BMP2-MPs (Supplementary Fig. 5). Differential expression of COL-X was observed between cells located in close proximity to injected BMP2-MPs within the PZ and CZ. Thus, whilst up-regulation of COL-X was negligible in PZ cells, COL-X expression was distinctly up-regulated in CZ cells in close proximity to injected BMP2-MPs in comparison to neighboring counterparts (Fig. 4). To evaluate expression of late osteogenic markers, IHC was performed using OC antibody. Despite detection of OC expression in bone collar and hypertrophic chondrocytes, expression of OC was not detected in cells with altered morphology in both PZ and CZ (Fig. 4).

In analyzed sections from samples with injected BMP2-MPs (n=6), the area of cells displaying morphological changes in chondroprogenitors or the zone of COL-I up-regulation was noted to be clearly limited to the proximity of injected MPs covering around 0.0105–0.101 mm² of surface area on each section (Supplementary Fig. 6). A macro was developed to measure the intensity of COL-I staining around injected MPs by image segmentation and three level thresholding.

Given the typically low-level COL-I expression in chondroprogenitors, image analysis of samples injected with blank MPs detected only low-intensity, and to a lesser extent, intermediate-intensity signals at the injection sites in the committed zone (CZ), with no detection of the high-intensity segment (Figs 5–6 and Supplementary Fig. 7).

In contrast, sections from samples injected with BMP2-MPs into the PZ exhibited a greater percentage of low- and intermediate-intensity COL-I staining around the injection sites compared to those around blank MPs. Furthermore, areas with high-intensity staining were observed, albeit at a lower proportion relative to the low- and intermediate-intensity signals (Figs 5–6 and Supplementary Fig. 8). In CZ, analysis of samples injected with BMP2-MPs revealed a significantly

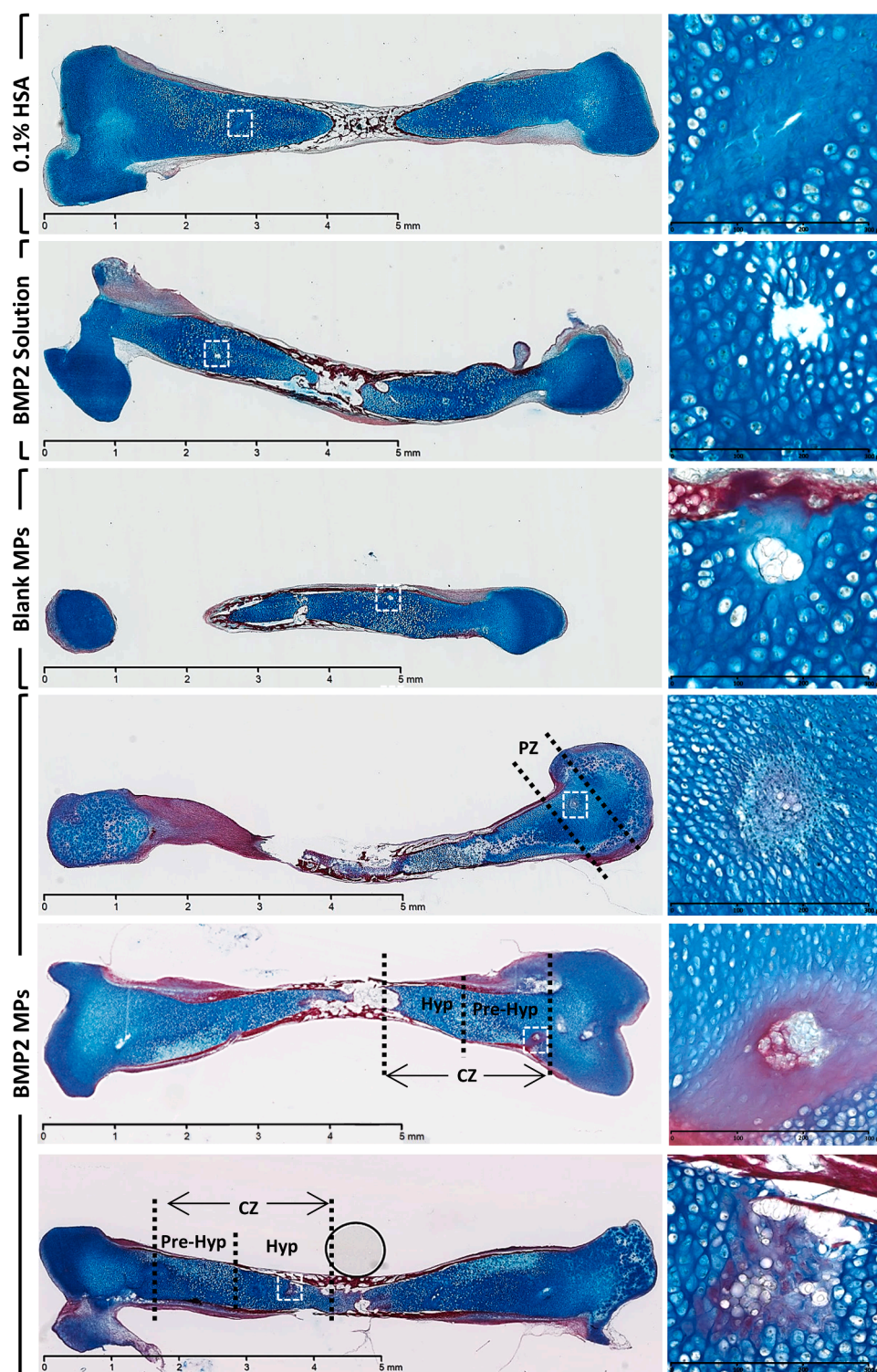


Fig. 2. Histological analysis of injected chick bones stained with Alcian blue/Sirius red staining with injection sites highlighted by arrows. Morphological changes in 1 % HSA, BMP2 solution and blank MPs controls were subtle and limited to changes in physical disturbance of tissue as a consequence of intervention. In bones injected with BMP2-MPs, morphological changes were observed in a zone of approximately 150 μm in diameter around injection site in proliferative zone (PZ) and deposition of collagen into ECM in samples injected in region contained prehypertrophic (Pre-Hyp) or hypertrophic (Hyp) chondrocytes, here described as committed zone (CZ).

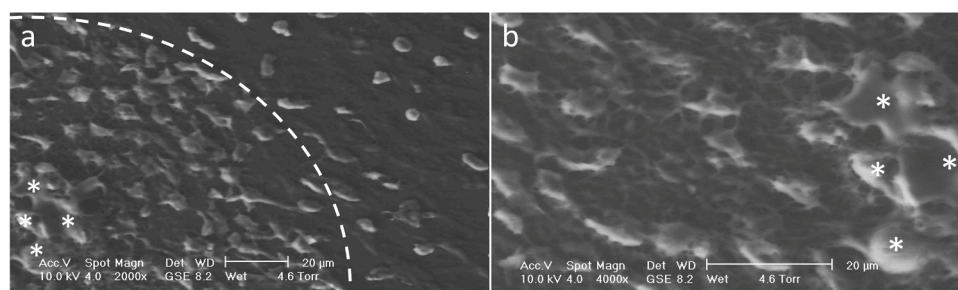


Fig. 3. ESEM analysis of injection sites revealed extensive morphological alteration in cells located in close proximity to injected BMP2-MPs (indicated with *). Whilst endogenous cells exhibited round morphology and were located apart from each other (a, outside of the hatched area), cells in close proximity to injected BMP2-MPs arranged in more compact fashion and exhibited an altered morphology with distinct cytoplasmic projections (a, inside hatched area and b).

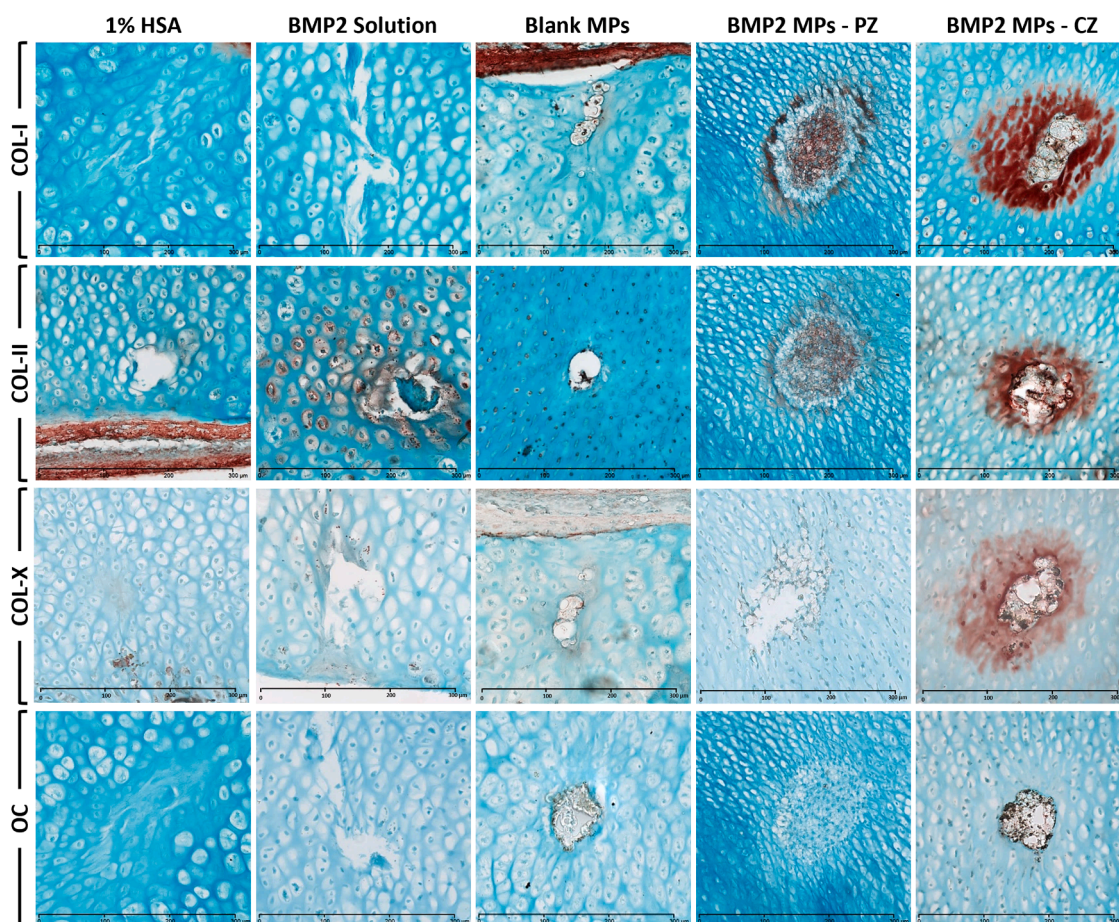


Fig. 4. Expression of both COL-I and COL-II was up-regulated in cells around injected BMP2-MP. Expression of COL-I and COL-II (particularly COL-I) was more pronounced in cell located in CZ. Expression of COL-X was negligible in the PZ cells while up-regulation of COL-X was higher in the CZ cells compared to neighboring counterparts. Expression of the late osteogenic marker OC was not detected. No extensive changes were observed in the level of expression of analyzed markers in cells located in close proximity to the injection sites compared with native cells in 1 % HSA, BMP2 solution and blank MPS control groups (Please refer to supplementary 5-8 for lower magnification illustration, COL-I, COL-II & OC scale bars: 300 µm, COL-X scale bars: 200 µm).

greater percentage of high-intensity COL-I staining compared to those injected in the PZ (Figs 5–6 and Supplementary Fig. 9).

3.3. FGF2-MPs injection

To evaluate the response of cells located in hyaline cartilage to the spatiotemporal release of FGF2, FGF2-MPs were injected into the chick embryonic bone following a similar approach as adopted for BMP2-MPs. Alcian blue/Sirius red staining of samples following injection of FGF2-MPs revealed that cells located in the PZ and in close proximity to

injected FGF2-MPs displayed morphological changes with the formation of extended cytoplasmic projections arranged in random formation (Fig. 7). ESEM analysis revealed longer cytoplasmic projections of increased size in PZ cells being exposed to FGF2 which were distinct from the morphology of cells being exposed to BMP2-MPs (Fig. 7). Further analysis of samples revealed differential regulation of COL-I and COL-II in chondrocytes located in both PZ and CZ in comparison with bones injected in the same area with BMP2-MP. While COL-II expression was highly up-regulated in comparison to native counterparts, expression of COL-I was not detected in cells exposed to FGF2-MPs. Similarly,

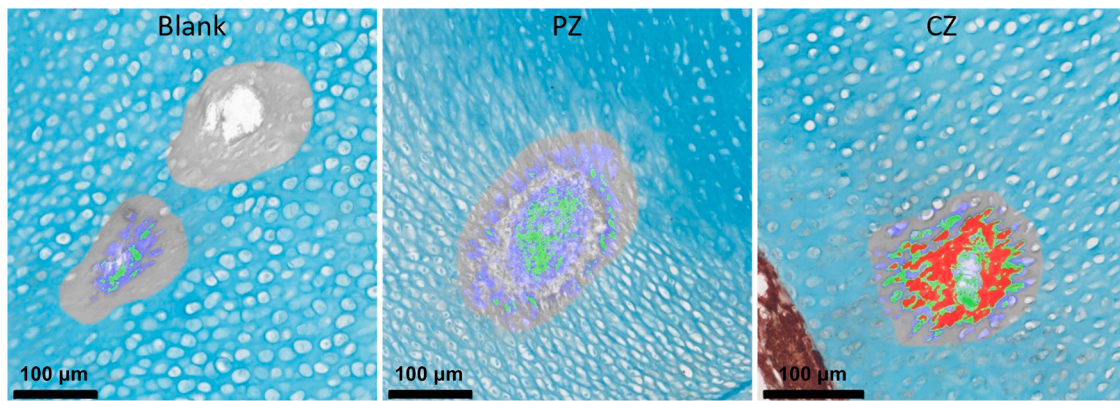


Fig. 5. Quantification of COL-I expression following exposure to BMP2 released from GF-loaded MPs based on three-level thresholding where intensity of pixels categorized into >151 (light DAB) as low (blue), 76–150 as intermediate (green) and <75 (dark DAB) as high in chick bone injected with Blank-MPs and BMP2-MPs in proliferative zone (PZ) and pre-hypertrophic zone (CZ).

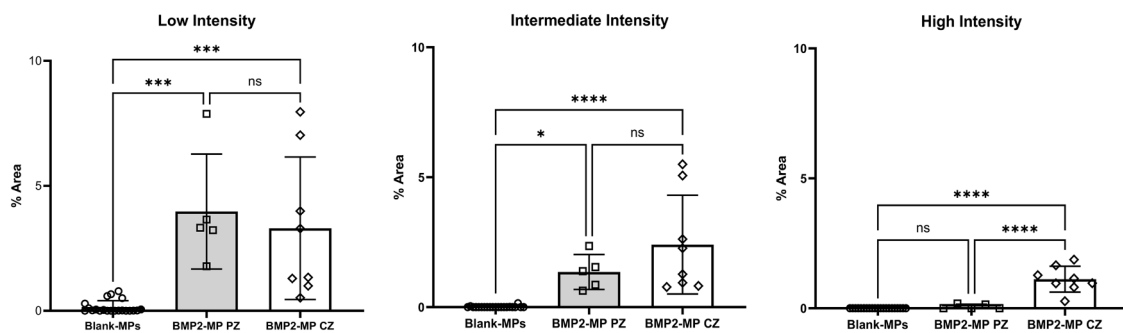


Fig. 6. Quantification of COL-I expression area in close vicinity of injection sites. Values represent mean $\pm$ SD for Ctrl (n=21), PZ (n=5) and CZ (n=8).

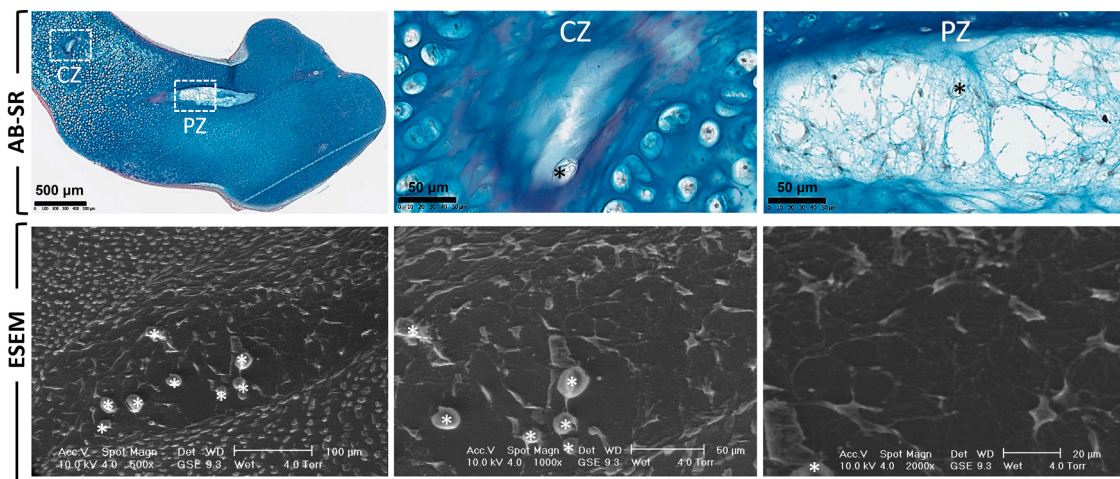


Fig. 7. Morphological changes was observed following transplantation of FGF2-MPs in sections stained with Alcian Blue/Sirius Red (AB-SR) which was more distinctive in PZ cells. The appearance and arrangement of morphologically changed cells were distinctive from PZ cells in close proximity to BMP2-MPs. No obvious alteration was observed in morphology of cells located in CZs. Following ESEM, longer cytoplasmic projections were observed in cells located in PZ following exposure to sustained release of FGF2 from MPs (indicated with *) and they re-arranged in a more dispersed fashion. These cells displayed a distinct morphology compared to cells exposed to BMP2.

up-regulation of only COL-II was observed in the CZ cells in close proximity to injected FGF2-MPs. A low level of COL-X expression was observed in CZ cells located around the injection site (Fig. 8).

4. Discussion

The current studies highlight the significant effect that spatio-

temporal regulation of growth factor signaling has on tissue formation. PLGA microparticles were employed to deliver and controllably release BMP2 and FGF2 in an *ex vivo* embryonic chick femur, which was used as a model of bone formation. Other groups have previously demonstrated the beneficial effects of controlled delivery and release of growth factors for regulating bone regeneration [24,39]. For instance, in a mouse digit amputation model, silica-based porous glass or agarose

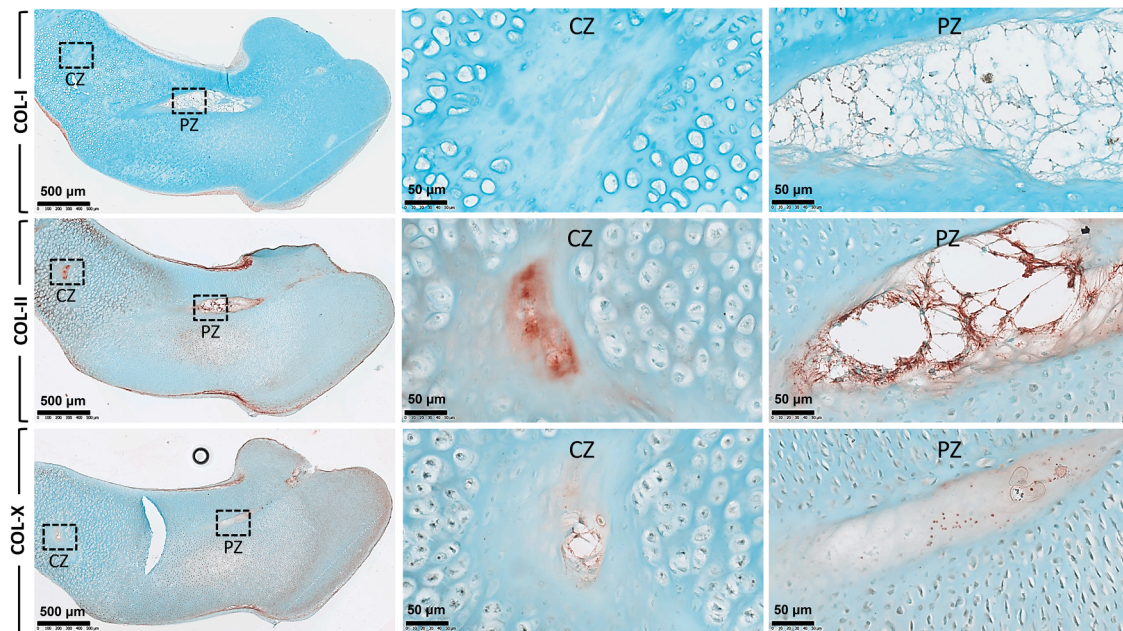


Fig. 8. Up-regulation of COL-II was observed in chondroprogenitors in the PZ and CZ in close proximity to the injection sites. No distinctive changes were observed in expression of COL-I and COL-X in cells located in close proximity to the injected FGF2-MPs compared with native cells.

beads were used for slow release of BMP2, which induced endochondral ossification at the digit amputation site [40,41].

The noted expression of COL-I in response to the microparticle delivered BMP2 indicated osteogenic commitment of chondroprogenitors and potential early onset of mineralization of matrix as described previously [42]. In contrast, the lack of OC expression, suggests limited osteoblast differentiation and maturation [43]. The lack of expression of OC and evidenced osteoblast maturation of skeletal populations may be a consequence of various factors including short exposure time and/or constant exposure to BMP2, which has been previously reported to delay terminal differentiation of hypertrophic chondrocytes [44,45]. In addition, inhibitory signals released from adjacent chondroprogenitors such as tenascin-w may play a role and inhibit further differentiation of exposed cells [46]. The observation that injection of BMP2 solution had a negligible effect on cells in close proximity to injection sites further substantiates the importance of a controlled-release strategy to induce the desired clinical effect and to improve reparative outcomes. As bacterial endotoxin has been shown to elicit cellular responses [47], endotoxin-free BMP2-loaded MPs (BMP2^{ORF}-MPs) were injected following a similar procedure to rule out the potential effects of endotoxin. Up-regulation of COL-I in cells in close proximity to injected BMP2^{ORF}-MPs suggested that the observed cellular response is a direct consequence of exposure to BMP2 released from MPs rather than bacterial endotoxin (Supplementary Fig. 5).

Fibroblast growth factors (FGFs) are important regulators of osteogenesis while their importance in bone tissue engineering and, critically, their precise role remains far from clear. Increased cellular proliferation and enhanced adipogenic differentiation [48] have been reported as well as inhibition of osteogenic differentiation following exposure to FGF2 [49,50]. Interestingly, synergistic effects of FGF2 and BMP2 on osteogenic differentiation of bone marrow-derived mesenchymal stem cells have been reported elsewhere [51,52]. Furthermore, Chiou and colleagues have shown FGF2 has mitogenic and chondrogenic effects on adipose-derived MSCs [53]. While it would be useful to have included a control where an FGF2 solution was injected to further enhance the data set generated, the noted lack of COL-I expression and up-regulation of COL-II following exposure to FGF2 in the current studies is rather in accordance with previously published data suggesting FGF2 as a negative regulator of osteoblast differentiation and enhancer of

chondrogenesis [49,50,53]. Moreover, the distinct regulation of COL-I and -II in cells located in close proximity to the injected BMP2-MPs in contrast to FGF2-MPs suggests a growth factor-dependent response to exogenous factors. These observations further emphasize the importance of a physiologically-relevant environment to delineate the effect of extrinsic inductive factors.

Previously, a modified *ex vivo* chick embryonic femur culture system was developed to study the effect of osteoinductive factors [34,36,54]. In addition, this organotypic model has been used as a critical bone defect model to study the effect of growth factor released from microparticles encapsulated in an alginate construct and implanted in a bone defect site created in the bone diaphysis [55–57].

To study the localized effect of spatiotemporal release of GFs, here we introduce a new approach by taking advantage of a developed and modified *ex vivo* chick embryonic femur culture system [34,36]. The lack of response in control groups injected with BMP2 solution indicates the importance of an efficient sustained release mechanism to fully harness GF potential. In the current study, growth factor-dependent responses of chondroprogenitors indicated the usefulness of this *ex vivo* system in evaluating the effect of GF released from MPs on tissue formation. In addition, this model has the potential to be used for the evaluation of more complex regimens based on the delivery of multiple growth factors. Therapeutic efficacy can be achieved, to a certain degree, following the delivery of a single exogenous factor, physiological systems typically necessitate a cascade of factors and the need for delivery platforms capable of providing spatiotemporal release of several, ideally inductive, factors to mimic aspects of the natural healing process [5]. To achieve such a level of sophistication and to evaluate the outcome, approaches have included high throughput screening [58,59], organ on a chip or microfluidic 3D cell culture devices [60,61], and mathematical modelling [62].

5. Conclusion

The routine treatment of critical-sized bone defects remains a challenging unmet need [63]. Enhanced understanding of the interaction of skeletal progenitor cells with chondro-, osteo-, and angiogenic factors is pivotal to the development of innovative efficacious strategies to mimic endochondral ossification using bone tissue engineering approaches

[64]. However, current *in vitro* assays are limited in their ability to provide all the requisite information.

The current results suggest that the organotypic chick embryonic bone provides a useful *ex vivo* tool to evaluate the activity of multiple growth factors within more physiologically relevant niches. This innovative approach offers much potential as an inexpensive physiologically relevant *ex vivo* model to bridge the gap between current *in vitro* assays and challenging and expensive *in vivo* critical-sized defect animal models.

CRedit authorship contribution statement

Hassan Rashidi: Writing – original draft, Visualization, Validation, Methodology, Formal analysis, Data curation. **Helen C. Cox:** Writing – review & editing, Resources, Methodology, Data curation. **Omar Qutachi:** Writing – review & editing, Resources, Methodology, Data curation. **Dale Moulding:** Writing – review & editing, Methodology, Data curation. **Lisa J. White:** Writing – review & editing, Resources, Methodology. **Emma L. Smith:** Resources, Methodology. **Janos M. Kanczler:** Writing – review & editing, Resources, Methodology. **Luis Rojo:** Writing – review & editing, Resources, Methodology. **Michael Rotherham:** Writing – review & editing, Resources, Methodology. **James R. Henstock:** Writing – review & editing, Resources. **Molly M. Stevens:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **Alicia J. El Haj:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **Richard O.C. Oreffo:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **Kevin M. Shakesheff:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **Felicity R.A.J. Rose:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Kevin M. Shakesheff is the current Director at Locate Bio Ltd. Molly M. Stevens has invested in, consults for (or is on scientific advisory boards or boards of directors) and conducts sponsored research funded by companies related to the biomaterials field; has filed patent applications related to biomaterials; and has co-founded companies in the biomaterials field. The other authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.actbio.2025.08.028](https://doi.org/10.1016/j.actbio.2025.08.028).

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