Bone induction at physiological doses of BMP through localization by clay nanoparticle gels

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Abstract

Bone Morphogenic Protein 2 (BMP2) can induce ectopic bone. This ability, which first motivated the widespread application of BMP2 in fracture healing and spinal arthrodesis has, more recently, been indicated as one of several serious adverse effects associated with the supra-physiological doses of BMP2 relied upon for clinical efficacy. Key to harnessing BMPs and other agents safely and effectively will be the ability to localize activity at a target site at substantially reduced doses. Clay (Laponite) nanoparticles can self assemble into gels under physiological conditions and bind growth factors for enhanced and localized efficacy. Here we show the ability to localize and enhance the activity of BMP2 to achieve ectopic bone formation at doses within the sub-microgram per ml range of concentrations sufficient to induce differentiation of responsive cell populations *in vitro* and at approximately 3000 fold lower than those employed in clinical practice.

Key Words: Smectites, Clay gels, Bone induction, Bone morphogenic protein, Drug delivery, Hydrogels

1. Introduction

Since early observations that co-administration of drugs with clay mineral-based antacids resulted in a dramatic reduction of systemic bioavailability, clay-protein interactions have been widely explored for drug delivery applications.[1] In particular smectite clays which swell and delaminate into individual particles, can host an array of potential interactions with organic molecules on particle surfaces or within inter-layer pores and inter-particle spaces through cation-exchange, hydrophobic interactions, hydrogen bonding, cation bridging, anion exchange and proton transfer. Clay-biomolecule interactions have been applied for delivery of drugs in tablet form through drying and compaction of clay-drug complexes, or else in polymer-clay nanocomposites developed for drug delivery and, more recently, tissue regeneration [1].

Laponite is a synthetic smectite with particles consisting of a central magnesium sheet sandwiched by two silica sheets giving rise to a disc shaped particle of approximately 25 nm diameter and 1 nm thickness [2]. A fraction of magnesium ions of the central sheet are substituted for lithium ions resulting in a negative charge across the surface of the Laponite particle when dispersed in water. At the particle edge the octahedral and tetrahedral sheets are disrupted and small, localised positive charges are generated by the absorption of hydroxyl groups. Dispersed particles self-organize via electrostatic interactions forming open, macroporous and reversible (thixotropic) gel (or glassy) states depending on clay and salt concentration [3].

We have demonstrated the ability to encapsulate bioactive molecules through the electrolyte induced gelation of pristine (as opposed to polymer composited) Laponite particles [4]. Drop-wise addition into solutions of physiological saline generated nanoparticle gel capsules displaying broad-spectrum affinity for proteins, and the ability to sustain and localize the effects of bioactive molecules *in vitro* and *in vivo*. Notably, Laponite clay gels were able to sustain the differentiation of viable bone marrow stromal cells and host growth factor mediated tissue invasion *in vivo*.

BMPs, originally isolated as the active factors underlying ectopic bone formation by demineralized bone matrix [5] have been the subject of intense research and development for their therapeutic use over more than three decades. Utilization of recombinant BMPs in the clinic however, has typically relied for efficacy on high doses of the protein administered in the order of milligrams. This stands in contrast to the micrograms of BMP per kilogram of demineralized bone matrix indicating the critical role the native extracellular matrix plays in controlling and sustaining the temporal-spatial regulation of BMP signaling.

In clinical practice around 50% of the BMP is released from the collagen sponge delivery device within 3-6 days [6]. As a consequence, large doses are required to stimulate bone formation [7]. A number of studies have demonstrated significant adverse effects following BMP use including: heterotopic ossification (bone formation at undesirable places), osteolysis, and swelling [7-9]. These adverse effects are typically attributed to poor localization to the target site compounded by the excess dose of BMP employed [7]. Development of an efficient BMP delivery vehicle

offers the potential to reduce the effective dose of BMP, facilitating fracture healing and fusion without precipitation of serious adverse effects.

Here we demonstrate the potential of self-organizing clay nanoparticle gels to safely harness BMP2 for bone induction.

2. Results

2.1. Clay gels localize BMP2 for enhanced effect in vitro

Following initial depletion studies indicating that only 15 minutes exposure of BMP2 solution (200 ng ml^{-1}) to clay gel capsules (3.75 mg ml^{-1} Laponite in total media volume) was sufficient to abolish the BMP2 dose-dependent up-regulation of alkaline phosphatase activity in C2C12 myoblasts [10] (**Fig. S1)**, we investigated the potential to achieve clay gel mediated localisation of BMP2 activity *in vitro*. Preliminary studies indicated fully hydrated clay gel films, successfully applied previously for endothelial cell culture [4], to be suboptimal for C2C12 myoblast culture (**Fig. S2**). As an alternative approach, we air-dried low volume (2.5µl) droplets of low concentration (10 mg ml^{-1}) Laponite clay suspensions upon tissue culture plastic (TCP) to generate spotted clay gel films upon rehydration in media. Uniformly confluent cultures of C2C12 cells were maintained across the clay gel film/TCP interface with no observed difference in cell density or morphology (**Fig. S3**).

To explore the ability of clay gel films to localise BMP2 activity, BMP2 was either encapsulated within clay suspension before formation of the film or added to the media in solution at the point of cell seeding. Consistent with depletion studies (Fig. S1), no increase in alkaline phosphatase activity was observed in C2C12 cells

cultured upon clay films with encapsulated BMP2, indicating a loss of BMP2 activity or availability as a result of clay incorporation and drying (**Fig. 1a**). However, when BMP2 was applied exogenously in solution (at the point of cell seeding), a significant ($P < 0.0001$) up-regulation of alkaline phosphatase activity was observed in the presence of clay gels (Fig. 1a) yielding a significant ($P < 0.0001$) 2.4-fold reduction in the EC_{50} of the C2C12 dose response to BMP2 (**Fig 1b**).

A significant ($P < 0.05$, one way ANOVA with Dunnett's) upregulation in the BMP2 mediated response was apparent at clay nanoparticle concentrations as low as 15 ng mm^{-2} clay film. The enhanced response to BMP2 was optimal at clay concentrations of 159 ng mm^{-2} and attenuated above 763 ng mm^{-2} – though still significant ($P < 0.01$, one way ANOVA with Dunnett's) up to 4.41 μg mm^{-2}, the maximum concentration tested (**Fig. 1c**). The enhancement in BMP2 activity was localised to cells in direct association with the clay film (**Fig. 1d**).

Though C2C12 response to BMP2 is cell density dependent, clay film mediated enhancement was independent of cell density (**Fig. S4**), confirming that localised increase in BMP2 concentration rather than increased cell adhesion mediated the enhanced response. Furthermore, clay film associated alkaline phosphatase activity was optimal at low concentrations (0-2 %) of foetal calf serum (FCS) growth supplement indicating the effect to be independent of co-factors within FCS (**Fig. S5**). Interestingly, while 5-10 % FCS was optimal in standard tissue culture conditions clay film enhancement was retarded at FCS concentrations above 5%, a likely consequence of competitive binding by excess protein. Modest preservation of soluble BMP2 activity was observed after 2 and 4 hrs incubation in the presence of clay films

(**Fig. S6**) consistent with an enhanced localised concentration of residually active BMP2 in media.

2.2 Clay gels localize BMP2 to functionalize bone graft for osteo-induction *in vivo*

To confirm the ability of clay gels to localise and enhance the activity of BMP2 *in vivo,* non-viable human trabecular bone graft (TBG) scaffolds were perfused with clay gels for implantation in a subcutaneous murine model. Preliminary *in vitro* studies confirmed the ability of clay gels to localise both an encapsulated and exogenously applied fluorescently labelled model protein within TBG scaffolds (**Fig. 2a**) and enhance the osteoinduction by exogenously applied BMP2 of C2C12 cells upon TBG surfaces (**Fig. S7**). BMP2 (1 μg) was thus encapsulated within the clay gel or, alternatively, applied in solution exogenously to the graft site in an equivalent volume of phosphate buffered saline (PBS) at the point of implantation. Using the same approach, BMP2 solution was also applied exogenously to the graft site in the absence of clay gel.

Co-registration of micro CT datasets obtained prior to, and after, 28 days subcutaneous implantation, demonstrated a significant (P <0.05) net increase in bone volume gain only when BMP2 was applied exogenously to TBG in the presence of clay gels (**Fig 2b-c**). This result confirms *in vivo* the observed ability of clay gels to localise and, thus, enhance exogenous BMP2 activity *in vitro*. Histologically, no new bone was observed in the absence of BMP2 and limited new bone formation was observed when BMP2 was delivered to TBG in the absence of clay gels (**Fig. S8**).

In all clay gel + BMP2 samples, extensive regions of histologically distinguishable new bone at various stages of development was observed. In clay gel with exogenous BMP2 treatments, bone formation was observed proximal to clay gels and in direct association with TBG surfaces interfaced with invading mesenchymal and endothelial tissue (**Fig. 3a**). In contrast, clay gel encapsulated BMP2 treatments consistently (6/6 samples compared to 2/6 exogenous and 0/6 BMP2 only) displayed regions of bone forming via the endochondral mode as evidenced by the association of new bone with proliferating and hypertrophic chondrocytes. In each case, chondrogenesis was intimately associated with the clay gels. Proliferating chondrocytes were observed embedded within regions of clay gel and residual gel fragments could be identified within more developed regions of osteochondral tissue (**Fig**. **3b**).

2.3 Ectopic bone formation at physiological doses of BMP through clay nanoparticle gel localisation

Numerous studies have explored the ability of various carrier materials to enhance ectopic bone formation (**Table S1**). Typically, carrier proficiency for reducing the effective dose is demonstrated at the range of 0.5-5 μg total dose and concentrations of 5-20 μg cm^{-3} implant volume (**Fig. 4a**). Following the confirmation above of the ability to enhance BMP2 activity via clay gel localization within this range (1 μg total dose; 20 μg cm^{-3} we investigated if clay gels could be applied to further reduce the effective dose of BMP2 to within the sub-microgram cm^{-3} concentrations sufficient to achieve osteogenic induction of responsive populations *in vitro*.

Thus, 500 ng (7 μg cm^{-3}) or 40 ng (0.57 μg cm^{-3}) doses of BMP2 encapsulated in clay gel were applied via perfusion to collagen sponges and implanted subcutaneously.

Collagen sponges perfused with BMP2-loaded alginate were implanted as controls as previous work has shown alginate hydrogels to mediate BMP2 delivery with greater efficiency than collagen alone [11]. Both alginate and clay gels sustained osteogenesis at 500 ng dose of BMP2 with mineralized tissue in both treatments apparent from two weeks (**Fig. S9**). No significant difference in incidence ($P = 0.14$; **Fig. S10**) or volume ($P = 0.34$) of mineralized tissue formation (per ml implant) was observed between clay gel and alginate carriers of 500 ng dose BMP2 after 8 weeks (**Fig. 4b-c**), though alginate carriers indicated higher mineral density relative to clay gels (**Fig. S11**), and histologically more developed bone formation (**Fig. S12**).

Only collagen scaffolds perfused with clay gels, not alginate, proved capable of sustaining osteogenesis at 40 ng doses of BMP2 (**Fig. 4b-c**) with significantly greater bone volume ($P = 0.003$; **Fig. 4c**) and incidence of bone formation ($P = 0.0003$; **Fig. S10**) apparent in clay gel vs. alginate treatments. Histologically, new mineralized collagen formation was apparent at both 500 ng and 40 ng doses of BMP2 in clay gels (**Fig. S12 and S13**). There was no significant difference in incidence or bone volume per ml implant, or histological assessments between 'low' and 'very low' dose in clay gel treatments.

3. Discussion

This study demonstrates that gels formed through the association of dispersed clay nanoparticles are able to localize BMP2 activity and substantially reduce the effective dose required to mediate osteogenesis compared to current methods.

Enhanced osteogenic differentiation *in vitro* was observed in cells growing on clay gel film surfaces in response to BMP2. Recent studies have demonstrated an intrinsic osteogenic effect of certain clays *in vitro* in bone marrow and adipose derived stromal cell cultures when dispersed within biomaterials or when applied directly to cells as a culture supplement [12-16]. For example Gaharwar *et al*. applied suspensions of Laponite to bone marrow derived stromal cultures and observed enhanced osteogenic differentiation independent of the osteogenic supplement dexamethasone [12]. In the current study, alkaline phosphatase activity in C2C12 cells seeded upon clay films was dependent on addition of BMP2 suggesting alternate pathways to be at play in clay nanoparticle osteo-induction of stromal cells. Additionally, our application of electrolyte induced particle aggregations as gels and films in contrast to dispersed clay platelets in polymer or media may also account for differences in intrinsic osteogenic effect.

Importantly, osteogenic enhancement *in vitro* was strongly localized to cells growing upon clay gel surfaces at low, otherwise sub-efficacious, ambient concentrations of growth factor. Recent attempts to generate localized 2D concentration gradients based on the natural affinity of BMPs to various substrates have relied on pre-incubation of the substrate in high concentration solutions under specific binding conditions followed by extensive washing to avoid burst release of unbound growth factor [17-19]. To our knowledge this is the first demonstration of the ability to enhance osteogenic responses through concentration of low amounts of exogenous BMP2 *in situ,* i.e. in the presence of cells. The simplicity of this approach allows considerable scope for functionalization of graft surfaces via clay film coating for localization of BMP2 and/or other growth factors such as VEGF [4].

Thus, we perfused non-viable trabecular bone graft material with clay gels for BMP2 localization, either by encapsulating BMP2 in clay gels prior to perfusion or by applying BMP2 exogenously in solution to TBG pre-perfused with clay gels at point of implantation. As with our *in vitro* studies, ectopic bone induction was dependent on BMP2 delivery with no bone induction observed in clay gel only controls. This does not, however, preclude the possibility, raised by the *in vitro* studies [13, 20] cited above, of a direct osteogenic effect by clay nanoparticles *in vivo,* which may still be detectable at orthotopic sites more conducive for osteogenesis.

Though, histologically, new bone formation was observed through application of BMP2 to bone graft in the absence of clay gels, no significant increase in bone formation was detectable over the background of bone loss via μCT analysis in the current study. This was the case even after co-registration of the graft pre- and post-implantation, due to the artefactual effects of graft movement which is likely accentuated with progressive loss of graft structure. Importantly, a significant net increase in bone volume gain compared to controls was apparent over this background when BMP2 was applied to bone graft pre-perfused with clay gels. It is not clear, however, the extent to which this observation is attributable to enhanced bone formation over reduced osteolysis when compared with BMP2 only samples – though both are clinically relevant advantages.

Interestingly, histological analysis suggested alternate modes of BMP-2 loading to favor alternate modes of ectopic ossification. Direct appositional bone formation appeared dominant where BMP2 was applied exogenously both in the presence and

absence of clay gel with bone formation observed in direct association with TBG surfaces interfaced with invading mesenchymal and endothelial tissue. In contrast, clay gel encapsulated BMP2 treatments typically displayed bone formation via the endochondral mode as evidenced by the association of new bone with proliferating and hypertrophic chondrocytes.

Injection of carrier-free BMP2 into muscle classically generates bone via endochondral ossification [21] whereas studies observing direct (intramembranous in the absence of a cartilage template) BMP2-induced bone formation note the phenomena to be carrier dependent [22, 23]. In particular, cell-biomaterial surface interactions, open pores facilitating vascularization and mechanical stability are cited as necessary conditions for BMP2 induction via the intramembranous mode . Given that carrier conditions were identical in clay gel loaded samples, the divergent responses observed can be attributed to distinct and different spatial distributions of BMP2 within the graft environment as a combined result of alternate loading regimes and clay gel localization. Thus, whereas exogenously applied BMP2 was largely concentrated, by clay gels [4], proximal to TBG surfaces (favoring direct ossification), encapsulated BMP2 remained localized within clay gels necessitating cell invasion and TBG surface-independent BMP2 induction. This observation thus underlines the facility of clay gels for localizing the activity of BMP2 *in vivo*.

To confirm that localizing BMP2 activity can reduce the effective dose, we assessed ectopic bone formation in clinical grade collagen sponges perfused with clay gels encapsulating 500 ng or 40 ng of BMP2. These treatments represent, respectively, doses near, or an order of magnitude below, the minimum effective doses for ectopic

bone induction recorded in the literature. While this range yielded a threshold effect in alginate gels with negligible bone formation at the lower compared with higher dose, clay gels proved able to sustain ectopic bone formation at both doses. This result provides strong evidence that efficient localization of active BMP2 is sufficient to achieve bone induction at physiologically relevant concentrations (taken as the magnitude of concentration sufficient for osteoinduction upon direct application of BMP2 to responsive cell populations *in vitro*). It is notable that a localized low dose remains effective outside of the osteogenic environment of an orthotopic site.

Interestingly, there was no significant difference in incidence or bone volume per ml implant, or histological assessments between 500 ng and 40 ng doses. This suggests that while able to localize BMP2 to induce bone at low doses, other aspects of the osteogenic microenvironment may be limiting in clay gel environments. Further studies as to the optimal mode of clay gel mediated BMP2 delivery are therefore warranted.

4. Conclusion

Despite decades of research into carriers able to enhance BMP2 efficacy, the doses required for reliable bone induction have remained an order of magnitude above the 10^{-4} mg ml^{-1} concentrations sufficient to induce osteogenic differentiation *in vitro*. Here we demonstrate that the sorptive properties of clay nanoparticle gels are able to localize BMP2 activity and substantially reduce the effective dose to within such physiologically relevant concentrations. These results offer potentially great significance to orthopedic surgery and encourage further explorations of self-organizing clay nanoparticle gels for sustaining regenerative micro-environments.

5. Experimental Section

Laponite preparation: Clay gels were prepared as described previously [4]. Briefly, Laponite XLG (BYK, Widnes, UK) was dispersed in distilled water to required concentration % weight Laponite per unit volume. The preparations were subsequently sterilized by autoclave and evaporated water replaced. To produce clay gel films for cell culture, 2.5 µl of 1 % Laponite (equivalent to 1.47 µg cm^{-2}) was placed on TCP and air dried for 2 hours at room temperature prior to cell seeding.

Cell culture and analysis of ALP activity: In vitro characterization of BMP2 localization by clay gels applied C2C12 myoblasts due to their well characterized dose dependent upregulation of alkaline phosphatase activity in response to BMP2. Unless stated otherwise C2C12 cells were seeded at $1x10^5$ cm^{-2} and cultured with D-MEM containing 1% Penicillin/Streptomycin, 2 % FCS. BMP2 was added at point of cell seeding, at 200 ng ml^{-1}. Following cell culture for 72 hours, cells were fixed in ethanol and alkaline phosphatase staining performed according to a standard protocol. Representative images were taken in bright field using Axiovert 200 microscope and Axiovision software V4.0. Cell Profiler software was used to calculate cell density and relative ALP staining intensity.

TBG functionalization: Donated human femoral heads were received from Southampton General Hospital with ethical approval. Cylinders of trabecular bone 4 mm in diameter were removed using a trephine. Samples were cut to remove any subchondral bone to form cylinders 4 mm in length. Bone sections underwent multiple washes in 5% Hydrogen Peroxide and saline to remove cells and fat. 20 µl of 1% Laponite was applied to a section of TBG and left to dry for 2 hours at 37 °C. Individual clay gel treated and untreated TBG sections were then immersed in 0.25 ml

C2C12 suspension containing 5×10^5–1×10^6 cells in DMEM containing 2% FCS + 200 ng ml^{-1} BMP2. The scaffolds were incubated with the cell suspension in a humidified incubator at 37 °C, 5% CO2 with gentle agitation to promote seeding for 2 hours before being transferred into fresh media (DMEM + 2% FCS + 200 ng ml^{-1} BMP2). After three days samples were fixed and stained for alkaline phosphatase activity as above. To visualize clay gel protein localization, TBG sections were perfused with 20 μl 2.5% Laponite premixed with 10 μg ml^{-1} fluorescein labelled bovine serum albumin (FITC-BSA) before incubation in media. Alternatively TBG was perfused with 2.5% Laponite alone before a 2 hour incubation in media containing 200 ng ml^{-1} FITC-BSA. For controls, TBG was perfused with FITC-BSA in PBS or incubated in 200 ng ml^{-1} FITC-BSA in the absence of Laponite. All TBG sections were washed in PBS for 2 hours, and representative images taken.

In vivo study design: Enhanced ectopic osteo-induction via BMP2 - clay gel interaction was tested using the murine subcutaneous implantation model in two separate studies. The first study assessed the effect of BMP2 delivery mode in association with clay gels on bone formation upon non-viable trabecular bone graft. The second study assessed the effect of clay gel delivery on the effective dose of BMP2 for ectopic osteo-induction. The second study utilized collagen sponges to address the sensitivity limitations of the first study due to the confounding effects of bone graft on micro-CT analysis.

A sample size of 6 was calculated for the first study following a study reporting bone mineral density following ectopic implantation of a BMP-2 laden hydrogel in rats [24] where mean bone mineral density of ectopic bone formed was 350 mg cm^{-3}, with

a standard deviation (σ) of 32. We assumed a useful clinical effect to be the doubling of the effect when BMP was delivered in the presence of Laponite, adjusted for 80% power (Zß =0.84), and significance at 95% (Zα/2=1.96) using the standard equation:

Sample size (n) = 2 σ2 (Zß+Zα/2)2/= (difference in mean) 2.

A sample size of 19 was used for the second study on the basis of data obtained from the first *in vivo* study where mean bone volumes were 20 mm^3 and 12 mm^3, in experimental vs. control groups respectively with a standard deviation of 11. In order to account for mice not completing the study we used a sample size of 24 for each test group.

In vivo study 1: Ectopic bone formation on clay functionalized TBG: All animal studies were conducted in compliance with ethical approval and in accordance with institutional guidelines. Nude mice were anaesthetized with an intra-peritoneal injection of a midazolam/fentanyl mix. A midline dorsal incision was made, 3 TBG cylinders were implanted on each side and wounds closed with clips. At point of implantation cylinders were perfused with either: 20 µl PBS (TBG alone), 20 µl 2.5% Laponite (TBG + clay gel), 20 µl PBS + 1 µg BMP2 (TBG + BMP2), 20 µl 2.5% Laponite + 1 µg BMP mixed in prior to application (TBG + clay gel + BMP2 premixed), or 20 µl 2.5% Laponite with 1 µg BMP added immediately following perfusion with Laponite (TBG + clay gel + BMP2 exogenous). Five mice were used in total, with n=6 samples for each of the 5 groups.

In vivo study 2: Ectopic bone formation by low dose BMP2: MF^{-1} mice were used, surgery and anesthesia was performed as above. A collagen sheet 4 mm in thickness was obtained from Medtronic. From this identical cylinders of 4 mm in diameter were prepared using a skin biopsy punch in a sterile environment. BMP solution of 1 μg μl^{-1} was mixed with 2% Laponite and 2% Alginate to produce gels containing 7 μg ml^{-1} and 0.57 μg ml^{-1} BMP. 140 μl of the gels was transferred to individual wells of a 96 well plate. Sponge cylinders were compressed and allowed to expand while submerged in the gel filled wells. Each mouse received 3 collagen cylinders containing high dose BMP gels on the left side and 3 with low dose BMP gels on the right, one mouse received 6 blank collagen cylinders as a control. In total 17 mice were used, with n=24 for each of the 4 groups: 1) Laponite 7 μg ml^{-1} BMP 2) Laponite 0.57 μg ml^{-1} BMP 3) Alginate 7 μg ml^{-1} BMP 4) Alginate 0.57 μg ml^{-1} BMP and n=6 in group 5) collagen only. Gels were made fresh for each individual mouse during induction of anesthesia, with BMP kept on dry ice until use. Volume of gel remaining following absorption was recorded for each individual cylinder and total volume of implant calculated as gel volume + non-porous volume of scaffold.

Micro CT: All CT scans were performed using Brunker Skyscan 1176, images were reconstructed using NRecon, and analyzed using CTAn software. Allograft cylinders were scanned prior to implantation with 50 kV voltage, 500 μA current, 0.5 mm Al filter and a pixel size of 9 μm. Following implantation allograft cylinders were removed and scanned again using the same settings. The same scan settings were used during the study of bone formation within the collagen cylinders with the exception that the pixel size was increased to 18 μm. Co-registration of pre and post

implantation TBG samples was conducted using CTAn software to identify volumes of bone gain vs. bone loss.

Histology: Fixed samples underwent decalcification in Histoline for 24 hours except those assessed for von kossa staining. Subsequently samples were dehydrated, embedded in wax and sectioned at 9 μm thickness. Alcian blue and Sirius red staining was performed according to standard protocols.

Statistical analysis: Statistical analysis was performed using GraphPad Prism 6.0. Unpaired t-tests were used to compare ALP activity on Laponite with TCP with statistical significance determined using the Holm-Sidak method when BMP concentration or cell seeding density were variables. BMP2 and clay nanoparticle concentration response curves were plotted as the log of respective concentrations and fitted using standard (sigmoidal and bell-shaped respectively) non-linear regression curves. The Log EC_{50} of the BMP2 dose response in the presence and absence of clay films was compared using a least sum of squares F-test against the null hypothesis of no difference. For BMP2 incubation time study a 2-way ANOVA test was performed with *P* values adjusted to account for multiple comparisons. Fisher's exact test was used to compare number collagen scaffolds demonstrating bone formation. The Kruskal Wallace ANOVA test was used to compare bone volume data on collagen and allograft scaffolds.

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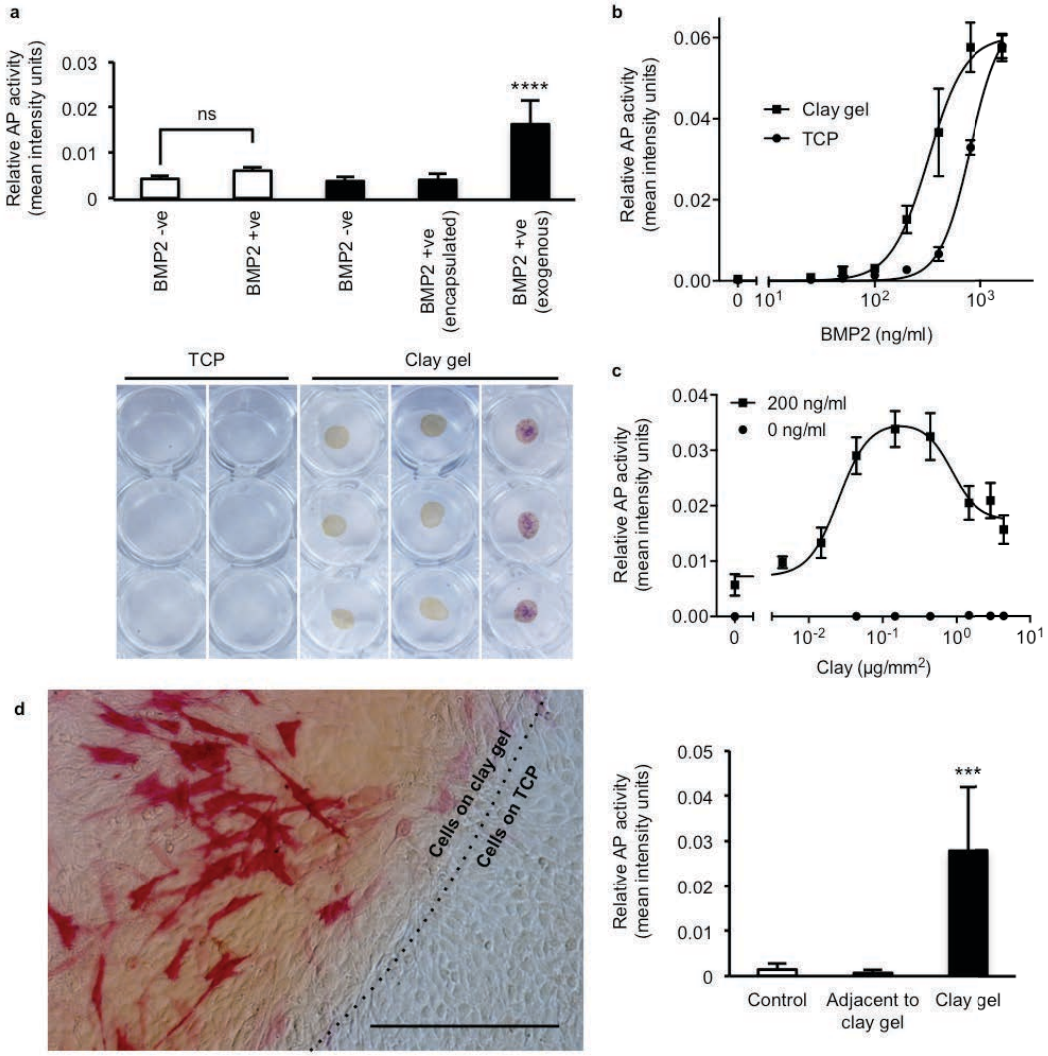

Figure 1. Clay gels localise BMP2 for enhanced effect in vitro. (a) Spotted and dried clay gel films enhanced the BMP2 induced osteogenic differentiation (increased alkaline phosphatase activity) of C2C12 myoblasts at doses (200 ng/ml) below that required under standard culture conditions. Enhanced alkaline phosphatase activity was observed when BMP2 was added exogenously to the media but not when encapsulated within clay gels. White and black bars correspond to TCP and clay gel samples respectively, *N*=6, **** indicates *P* <0.0001, one way ANOVA with Tukey's multiple comparisons test. (b) Spotted and dried clay gel films significantly (*P* <0.0001, Least sum of squares F-test for a sigmoidal dose response (variable slope) curve) decreased the EC_{50} of BMP2 induced osteogenic differentiation of C2C12 myoblasts (325.9, 95% *CI* = 276.4 - 384.2 ng ml^{-1} BMP2) in comparison to standard TCP substrates (791.6, 95% *CI* = 746.8 - 839.1 ng ml^{-1} BMP2). (c) Effect of clay concentration in spotted films on enhancement of C2C12 myoblast osteogenic differentiation is dependent on BMP2 (*P* <0.0001, one way ANOVA) and conforms to a bell-shaped dose response curve (R^2 = 0.9173, EC_{50} = 0.025; 95% *CI* = 0.016 - 0.038 and IC_{50} = 1.143, 95% *CI* = 0.436-1.751 µg mm^{-2}). Error bars = *SD*. *N*=3. Enhanced alkaline phosphatase activity was localised to cells growing in direct association with clay gel surfaces. Scale = 200 µm. Error bars = *SD*, *N*=3, *** indicates *P* <0.001, one way ANOVA with Tukey's multiple comparisons test.

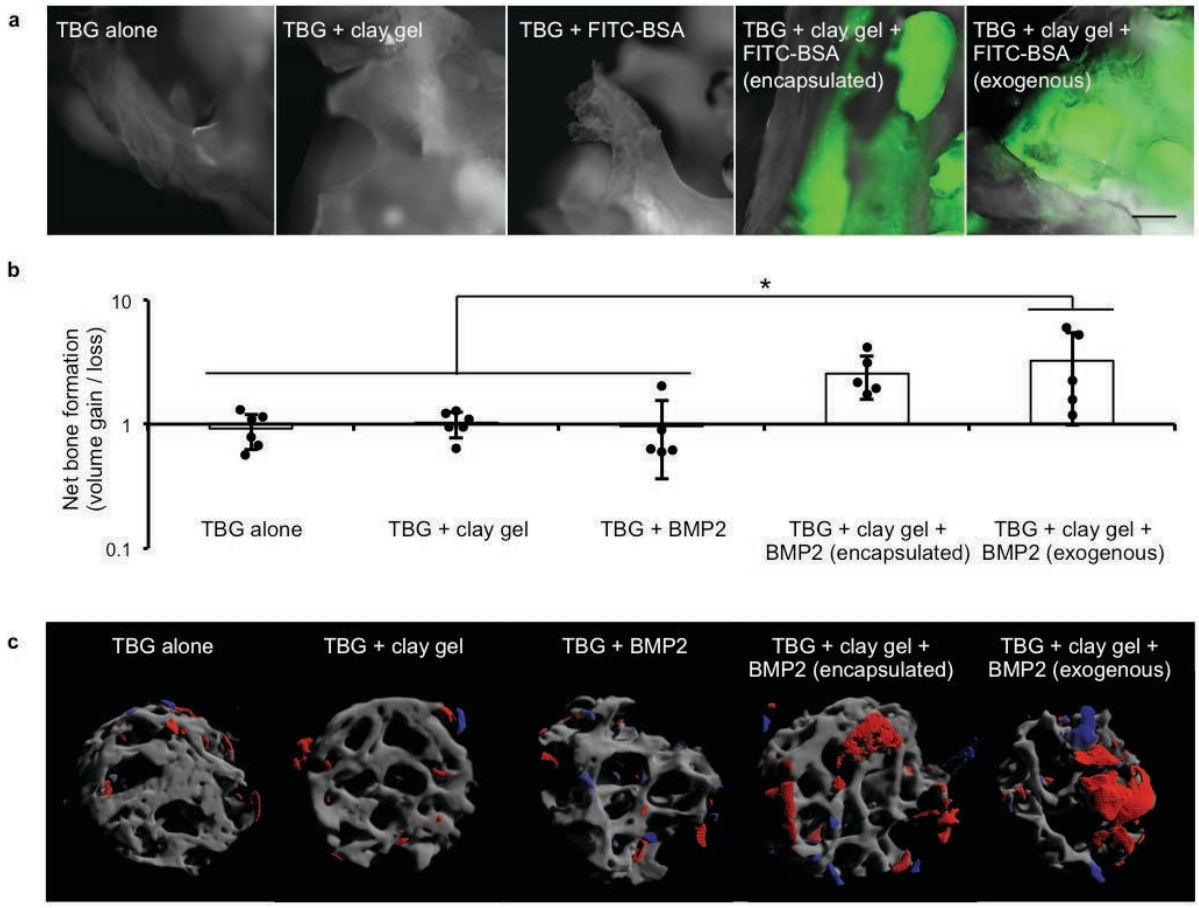

Figure 2. Clay gels functionalize non-viable trabecular bone graft to enhance BMP2 induced ectopic bone formation *in vivo.* (a) *In vitro,* clay gels localized labelled protein (FITC-BSA) within trabecular bone graft (TBG) when encapsulated or (unlike TBG + FITC-BSA) added exogenously in PBS. Scale = 200 μm. (b & c). *In vivo*, μCT analysis detected significantly enhanced bone formation only when BMP-2 was delivered exogenously to TBG perfused with clay gel. Bone formation was assessed via co-registration of TBG pre- and post- implantation. Net bone formation was calculated as bone volume gain over bone volume loss (indicated red and blue respectively in c). Error bars = SD, N=5 (or 6 for TBG and TBG + clay gel) for each group, * indicates $P < 0.05$, one-way ANOVA with Tukey's multiple comparisons test.

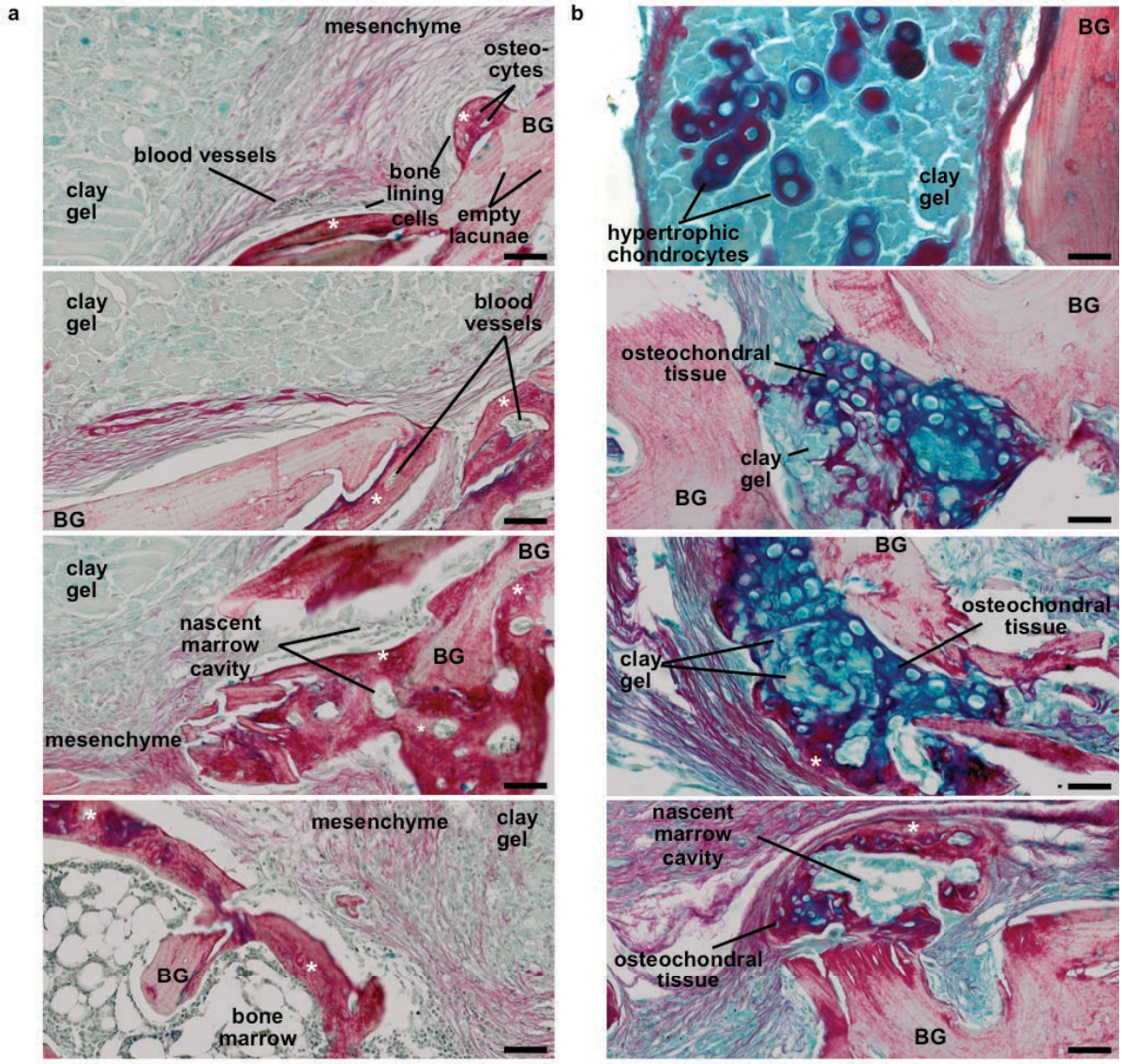

Figure 3. Alternate modes of BMP-2 loading in clay gels induce intramembranous or endochondral modes of ectopic bone formation. (a) Direct, appositional bone formation was observed upon bone graft surfaces (and enhanced by clay gel) predominantly in response to exogenously applied BMP-2. Nascent bone, morphologically distinguishable from bone graft can be observed in direct association with graft material interfacing mesenchymal and endothelial tissue in close proximity but adjacent to clay gels. More developed regions of bone with developing and developed bone marrow cavities could observed in certain regions still in association with bone graft and proximal to clay gel material. (b) Endochondral ossification, localized within clay gels, was dominant in response to encapsulated BMP-2. Proliferating and hypertrophic chondrocytes are observed embedded within clay gels suggesting cell invasion preceded BMP2 mediated responses. In regions of more developed osteochondral tissue, fragments of clay gel can be observed embedded within tissue. * = New bone; BG = Bone graft. Scale = 50 μm.

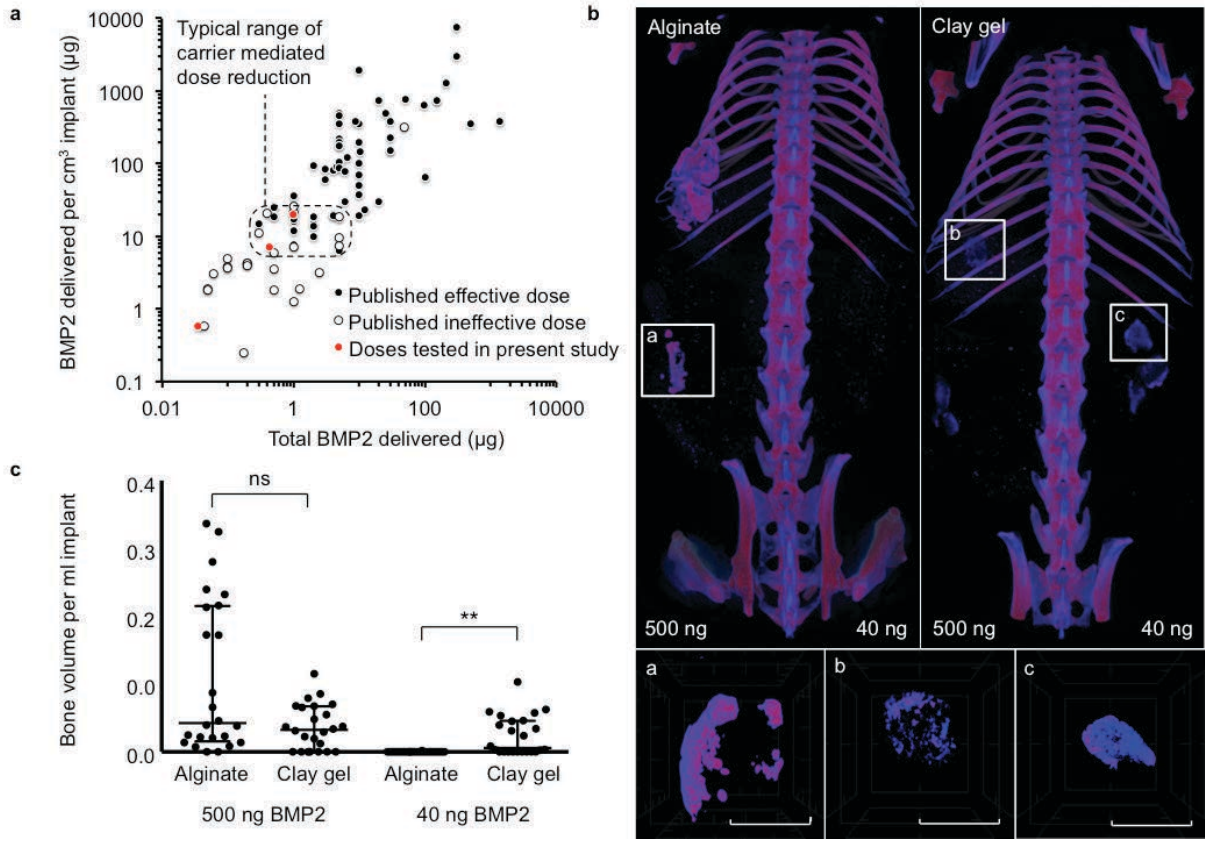

Figure 4. Clay gels uniquely sustain ectopic bone formation at low doses of BMP2. (a) Effective and ineffective BMP2 doses tested for ectopic bone induction reported in literature. Total dose against dose per cm^3 implant derived from 67 identified studies testing 72 carrier materials for BMP2 induction of ectopic bone (subcutaneous or muscle) across a range of animal models. For list of studies see **Table S1**. (b) False color µCT reconstructions of ectopic bone through Laponite or alginate delivery of encapsulated 500 ng and 40 ng doses of BMP2 (n=24) perfused through a collagen sponge. Scale 2.5 mm. (c) Laponite delivery sustained significantly higher ectopic bone formation at 40 ng doses of BMP2 compared with alginate control. N=24, ** indicates P <0.01, Kruskal-Wallis with Dunn's multiple comparisons test. Graph plots median with interquartile range.

Supporting Information

Bone induction at physiological doses of BMP through localization by clay nanoparticle gels

David M.R. Gibbs, *Cameron R.M. Black*, *Gry Hulsart-Billstrom*, *Pujiang Shi*, *Edoardo Scarpa* and *Richard O.C. Oreffo**, *Jonathan I. Dawson**

Supplementary figures

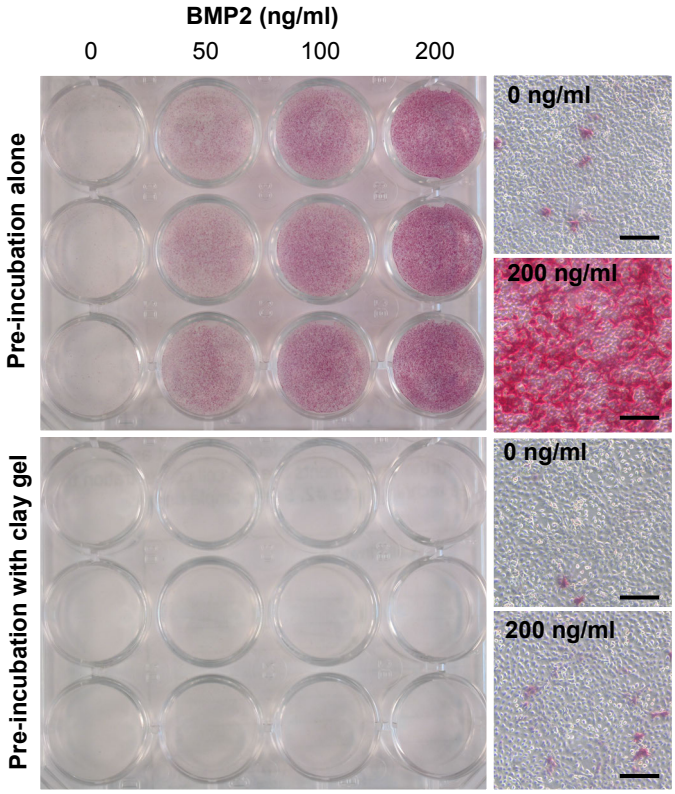

Figure S1. Clay gel mediated BMP2 depletion. Alkaline phosphatase activity of C2C12 myoblasts in response to doses of BMP2 pre-incubated in the presence or absence of clay gel capsules (3.75 mg ml^{-1} Laponite). Scale 200 μm.

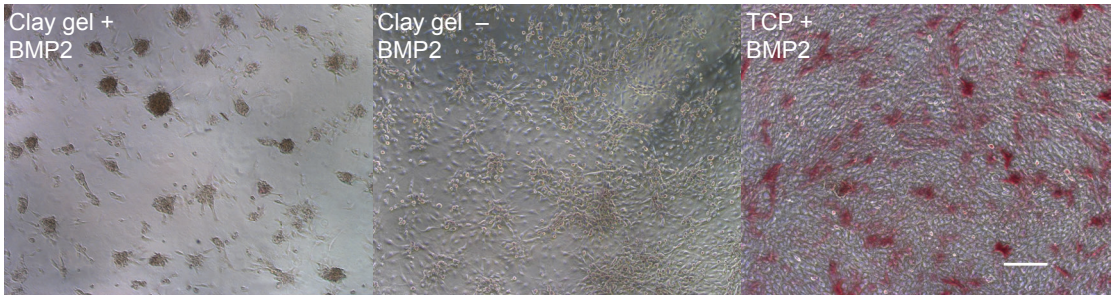

Figure S2. C2C12 myoblast response to hydrated clay gel films. Clay gel films were suboptimal for C2C12 culture due to a propensity of C2C12 myoblasts to disassociate from the soft gel substrate to form cell aggregates upon exposure to BMP2 (200 ng/ml). This behaviour is not observed on tissue culture plastic (TCP). Scale 200 μm.

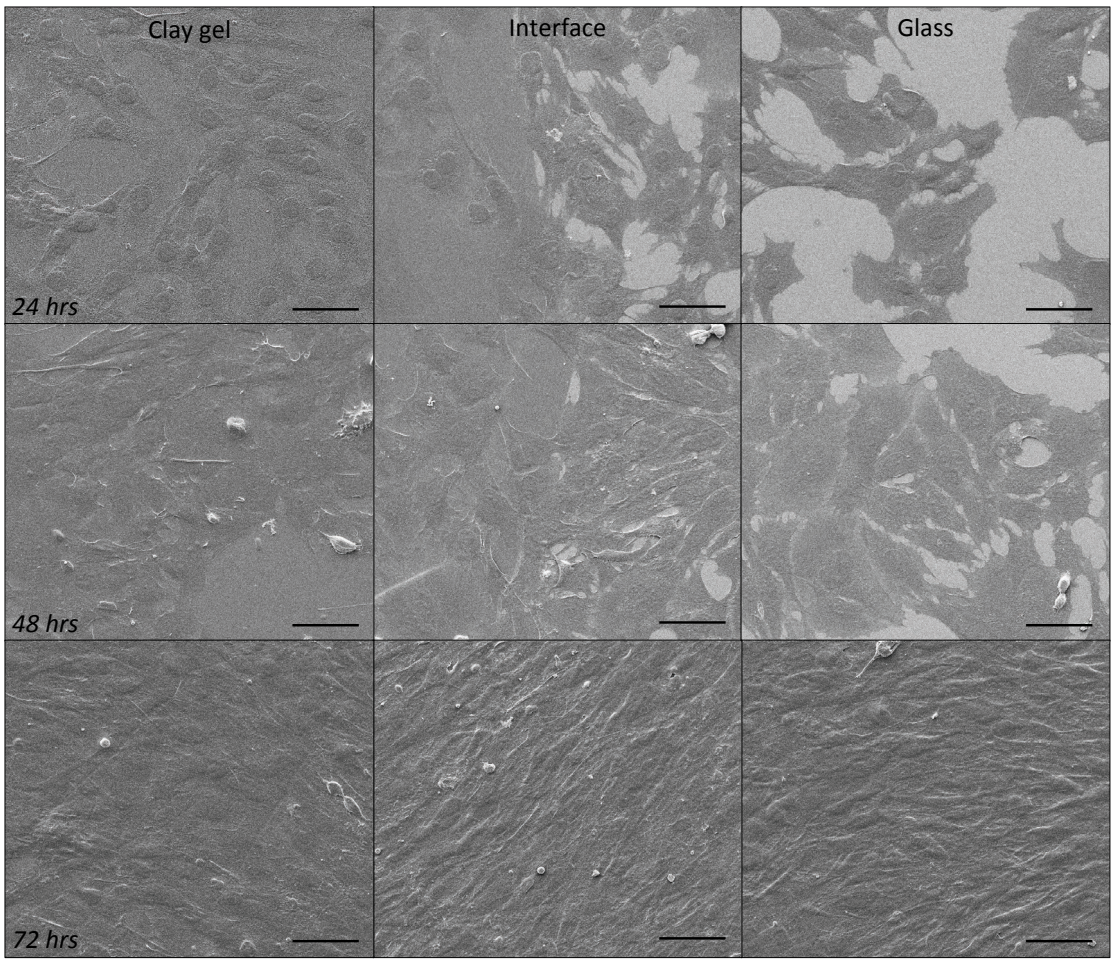

Figure S3. SEM micrographs of C2C12 myoblasts on clay gel films compared to glass control surfaces. C2C12 myoblasts were cultured on spotted and dried films of clay gel. No differences in cell density or morphology were observed on clay gel treated surfaces compared to control surfaces. Scale 50 μm.

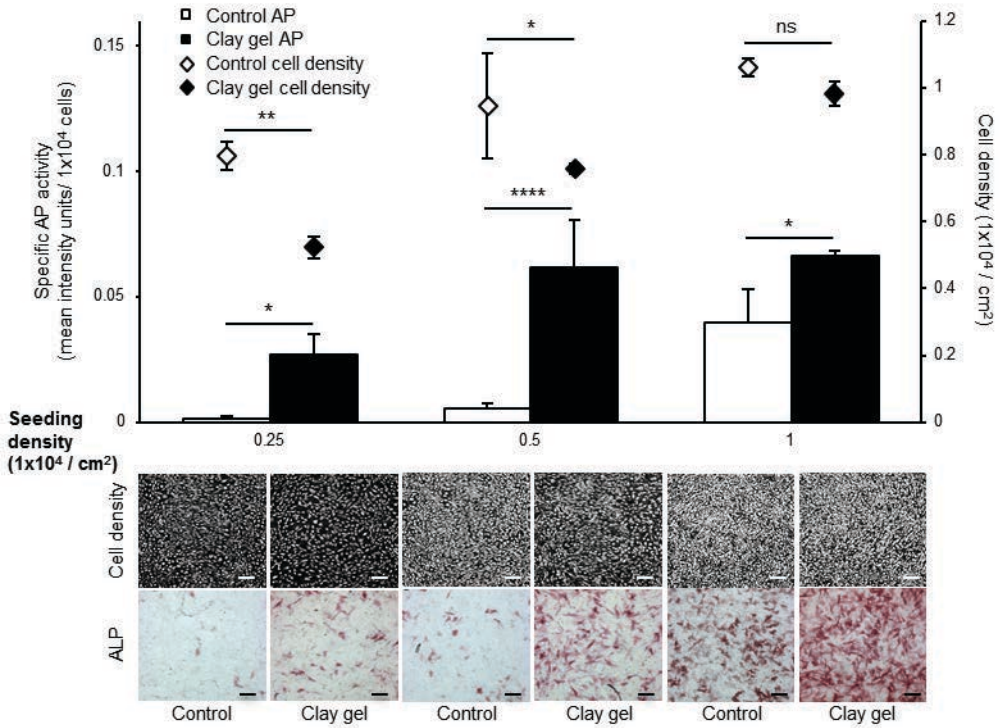

Figure S4. Clay gel enhancement of C2C12 osteogenic differentiation is not cell density dependent. Spotted and dried clay gel films enhanced the BMP2 induced osteogenic differentiation of C2C12 myoblasts (increased alkaline phosphatase activity) independently of the cell density dependent increase in BMP2 mediated osteogenesis. At 0.25 and 0.5 x10^4 cm^{-2} seeding densities final cell densities after three days were lower on clay gel surfaces compared with control surfaces indicating reduced proliferation. Scale 200 μm. Error bars = SD

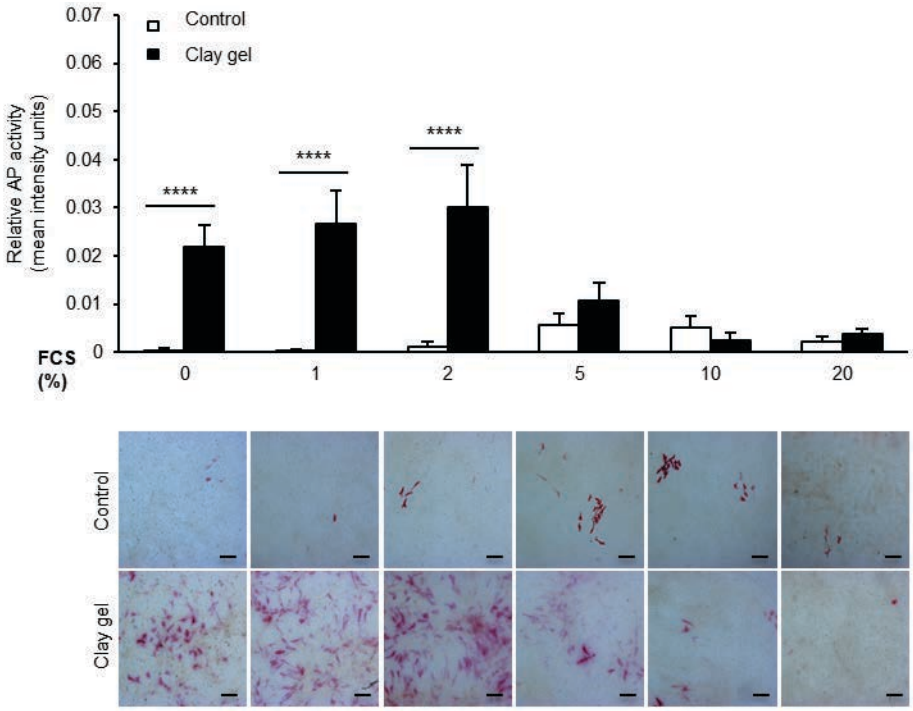

Figure S5. Clay gel enhancement of C2C12 osteogenic differentiation is attenuated at increased foetal calf serum (FCS) concentrations. Spotted and dried clay gel films enhanced the BMP2 induced osteogenic differentiation of C2C12 myoblasts (increased alkaline phosphatase activity) at FCS concentrations of 2% and below. Optimal osteogenesis in control conditions was observed and 5-10% FCS. Scale 200 μm. Error bars = SD

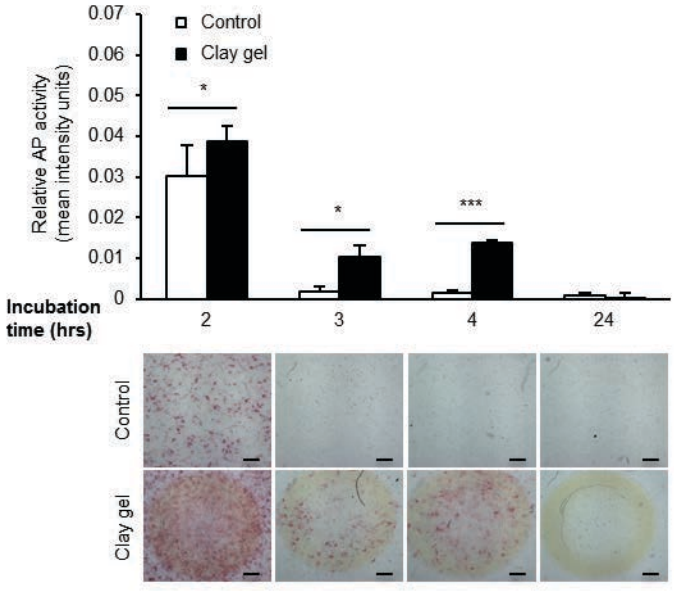

Figure S6. Clay gel enhancement of C2C12 osteogenic differentiation is preserved over 4 hours. Spotted and dried clay gel films sustained the BMP2 (400 ng/ml added exogenously to media) induced osteogenic differentiation of C2C12 myoblasts (increased alkaline phosphatase activity) after 4 hours in contrast to control surfaces upon which BMP2 activity was lost before 3 hours incubation. Scale 200 µm. Error bars = SD

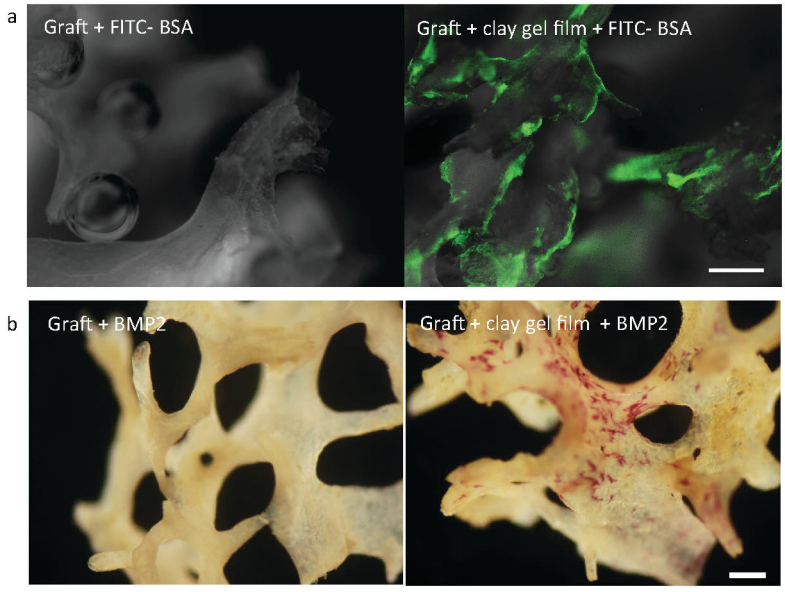

Figure S7. Clay gel films can impart bioactivity to graft material. a. Clay films generated by rinsing bone graft in 1% Laponite suspensions before drying localised exogenously applied FITC-BSA. Scale 200 µm. b. Using the same approach, clay gel films enhanced the osteogenic differentiation of C2C12 myoblasts (increased alkaline phosphatase activity) seeded on bone graft following culture in BMP2 containing media (200 ng ml^{-1}) compared to cells seeded on bone graft without clay gel film. Scale 250 µm.

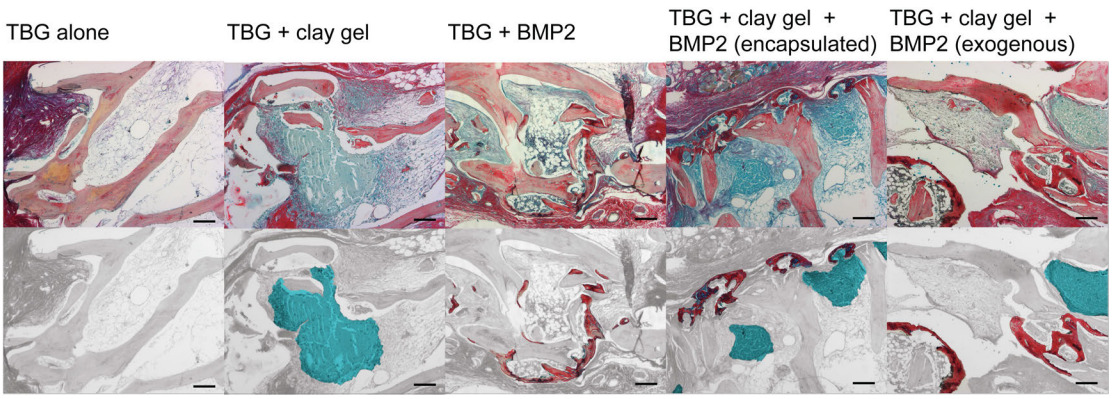

Figure S8. BMP2 induced bone formation on TBG. Histological assessment revealed new bone (morphologically distinguishable new bone formation highlighted in lower panel) only in BMP2 treatment groups. Residual clay gel is highlighted blue in lower panel. Scale bar = 200um.

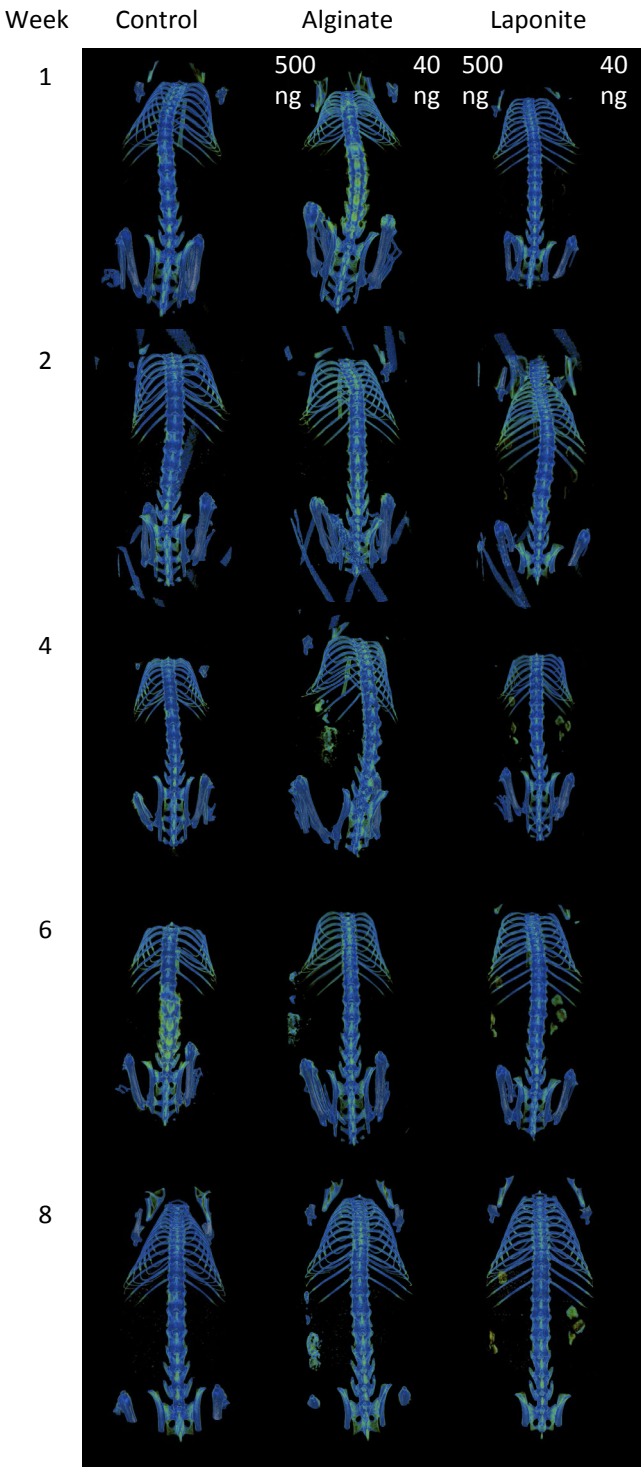

Figure S9. μCT enabled longitudinal analysis of ectopic bone formation at week 1, 2, 4, 6 and 8. Alginate and Laponite gels loaded with 500 or 40ng doses of BMP2 were perfused through a collagen sponge scaffold and implanted subcutaneously. 500 ng loaded gels were implanted in the left, 40 ng loaded gels were implanted in the right hand sub-cut region. Controls consisted of collagen scaffold alone.

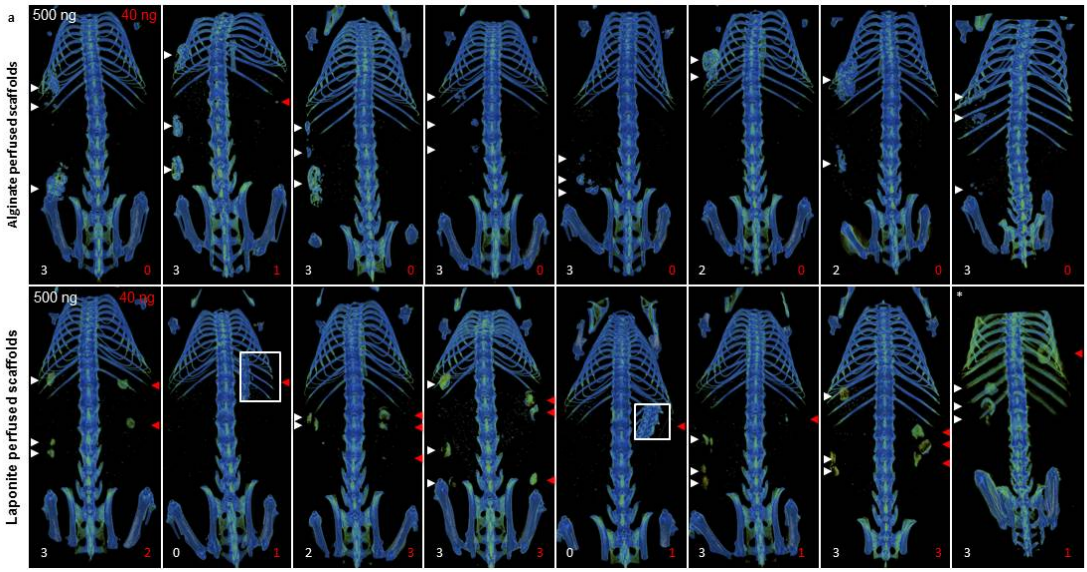

Figure S10. Incidence of ectopic bone formation at week 8. Alginate and Laponite gels loaded with 500 or 40 ng doses of BMP2 were perfused through a collagen sponge scaffold and implanted subcutaneously. 500 ng loaded gels were implanted in the left, 40 ng loaded gels were implanted in the right hand sub-cut region.

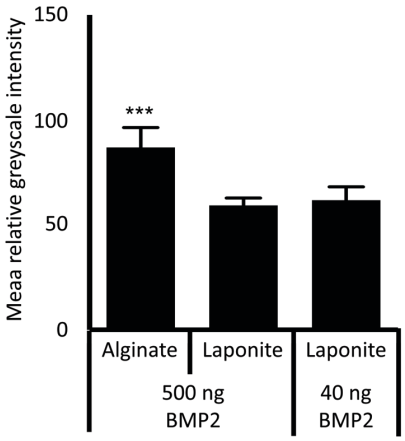

Figure S11. Mean relative greyscale intensity of thresholded ectopic mineralised tissue reveals greater bone mineral density in alginate (with 500 ng BMP2) compared with Laponite gels. Error bars = SD

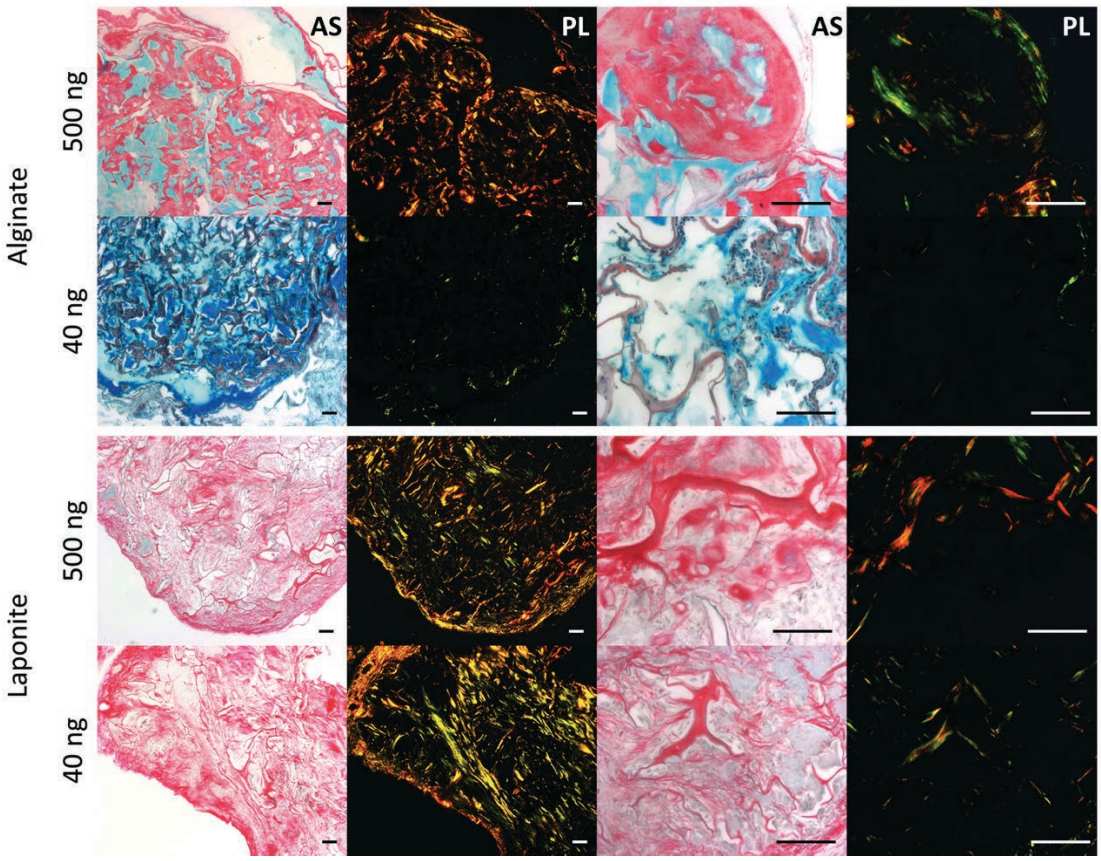

Figure S12. Sirius red and Alcian blue staining (AS) and polarised light microscopy (PL) confirm new bone formation in alginate (stains deep blue) loaded with 500 ng but not 40 ng doses of BMP2. Spicules of osteoid in association with clay gels (stains pale blue) could be observed with both 500 ng and 40 ng doses of BMP2. Scale = 100 µm.

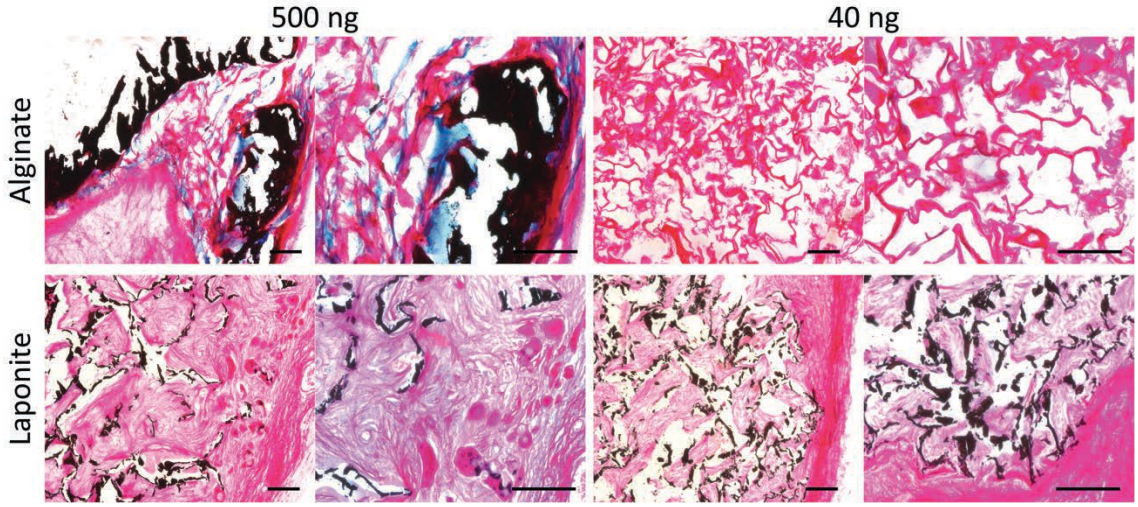

Figure S13. Von kossa staining confirms mineralised tissue at 500 ng BMP2 doses in alginate and 500 ng and 40 ng BMP2 doses in Laponite. Scale = 100 µm.

Supplementary Table 1.

Table S1. Literature review of effective and ineffective BMP2 doses tested for ectopic bone induction with various carriers

Minimum effective dose [µg][a)]	Ineffective doses tested [µg]	Minimum effective conc. [µg /cm3][b)]	Carrier[c)]	Site	Animal	Ref
No bone	0.18	n/a	Alginate +/-gelatin	SubCut	Mouse	[1]
No bone	0.2	n/a	Alginate + Bone marrow stromal cells	SubCut	Mouse	[2]
No bone	1	n/a	HA	SubCut	Rat	[3]
5 [d]	1, 2.5	6.4	Collagen sponge	SubCut	Rat	[4]
1		7.7	PEG-fibrinogen	SubCut	Rat	[5]
2		10	Collagen gel + BMP-binding peptide	SubCut	Rat	[6]
1	0.5	12	Gelatin	SubCut	Mouse	[7]
2	1, 0.5	14	PDLA-DX-PEG	Muscle	Mouse	[8]
0.3	0.06	15	HA/collagen /alginate	Muscle	Rat	[9]
2		15	CaP	SubCut	Rat	[10]
1		16	Inactive DBM + BMP binding protein	SubCut	Rat	[11]
2		16	TCP	Muscle	Rat	[12]
1		17	Heparin/ PLGA in fibrin	SubCut	Rat	[13]
5		18	PEG Hydrogel	Muscle	Mouse	[14]
0.5	0.05, 0.1, 0.3	18.5	HA	SubCut	Rat	[15-17]
2		18.5	PDLA-DX-PEG / TCP	Muscle	Mouse	[18]
0.5	0.05, 0.1	19	CaP	SubCut	Rat	[19]
4		19	PLGA capsules	SubCut	Rat	[20]
10	5	19	PDLA / PEG block copolymers	Muscle	Mouse	[21]
1		20	Heparin /PLGA	Muscle	Rat	[22]
4		20	Hyaluronan	SubCut	Rat	[23]
4		20	Hyaluronan	SubCut	Rat	[24]
1	0.2	21	Hyaluronan	Muscle	Rat	[25]
12		23	PLGA/Gelatin + Calcium Phosphate	Muscle	Dog	[26]
1		24	Type I Collagen	Muscle	Mouse	[8]
1		24	PLA-DX-PEG	Muscle	Mouse	[8]
0.5	0.1	25	PDLA / PEG	Muscle	Mouse	[21]
0.5	0.4	26	Collagen sponge (+ collagen-binding BMP2)	Muscle	Rat	[27]
6		30	Hyaluronan	Muscle	Rat	[28]
20	5, 1.25	30	TCP	Muscle	Mouse	[29]
1		36	Heparinised alginate	SubCut	Mouse	[30]
10	5, 0.5	37	Type I Collagen sponge	Muscle	Rat	[31]
10		50	Heparin-conjugated fibrin	SubCut	Mouse	[32]
3		60	BSA nanoparticles/polyethyl-enimine	SubCut	Rat	[33]
100		64	Gelatin Sponge + sulphated chitosan	Muscle	Mouse	[34]
10		71	Calcium phosphate cement	SubCut	Rabbit	[35]
6		78	Gelatin + PLGA microspheres	SubCut	Rat	[36]
4		80[e)]	Freeze dried collagen	Muscle	Mouse	[37]
3		83	Heparinised Gelatin	SubCut	Mouse	[38]
5		88	Collagen sponge	SubCut	Rat	[39]
4.5		90	Bacterial Cellulose	SubCut	Rat	[40]
2		95	Lyophilised Type I Collagen	Muscle	Rat	[41]
5		100	Chitosan Nanoparticles	Muscle	Mouse	[42]
10		100	Matrigel +/- gelatin	SubCut	Rat	[1]
5	0.2, 1	106	Collagen sponge	Muscle	Rat	[25]
5		106	Hyaluronan hydrogel	Muscle	Rat	[43]
6.5		123	PLGA/ PPF/gelatin composites	SubCut	Rat	[44, 45]
10.3		147	CaP	SubCut	Rat	[46]
30		150	Hyaluronan hydrogel	SubCut	Rat	[47]
30		150	Hyaluronan hydrogel	SubCut	Rat	[48]
5		179	Collagen sponge	SubCut	Mouse	[49]
5		179	Heparinised collagen sponge	SubCut	Mouse	[50]
5		200	HA	SubCut	Rat	[51]
10		200	Antibody conjugated collagen scaffold	SubCut	Rat	[52]
5		219.3	Lyophilised collagen	Muscle	Rat	[53]
500		350	PLGA	Muscle	Rat	[54]
10		353	CaP cement	Muscle	Rabbit	[55]
5		357	Collagen sponge + dextran	Muscle	Rat	[56]
30		375	Heparin/DBM	SubCut	Rat	[57]
8.7		378	Titanium mesh	SubCut	Rat	[58]
1400		387	PLGA Capsules	SubCut	Rat	[59]
5		455	HA	SubCut	Rat	[60]
5		500	Silk fibroin particles	SubCut	Rat	[61]

25		500	Collagen/DNA	SubCut	Rat	[62]
96	48	636	PDLA	Muscle	Rat	[63]
20		741	HA/TCP	SubCut	Rat	[64]
150		750	Chitosan hydrogel	Muscle	Rat	[65]
150		750	Hyaluronan hydrogel/ Chitosan hydrogel	Muscle	Rat	[66]
50		769	TCP	Muscle	Mouse	[67]
208		1300	DBM (collagen-binding BMP2)	SubCut	Rat	[68]
10		1908	HA Granules	Muscle	Rat	[69]
300		3000	Insoluble bone matrix	SubCut	Rat	[70]
300		7692	HA	SubCut	Rat	[71]

[a)]Effective dose considered as detectable bone induction over negative control. [b)]Where not stated in study, BMP2 concentration calculated per cm^3 implant. [c)]BSA, bovine serum albumin CaP, calcium phosphate; DBM, demineralized bone matrix; DX, p-dioxanone; HA, hydroxyapatite; PDLA, poly-d,l-lactic acid; PEG, poly(ethylene glycol); PLGA, poly(lactic-co-glycolic acid); PPF, poly(propylene fumarate); TCP, tri-calcium phosphate. [d)]Bone induction achieved at lower concentration through ultrasound not included in this analysis. [e)]Volume estimated from μCT ROI.

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