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Translating the role of osteogenic-angiogenic coupling in bone formation: Highly efficient chitosan-pDNA activated scaffolds can accelerate bone regeneration in critical-sized bone defects

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ABSTRACT

The clinical translation of bioactive scaffolds for the treatment of large segmental bone defects has remained a challenge due to safety and efficacy concerns as well as prohibitive costs. The design of an implantable, biocompatible and resorbable device, which can fill the defect space, allow for cell infiltration, differentiation and neovascularisation, while also recapitulating the natural repair process and inducing cells to lay down new bone tissue, would alleviate the problems with existing treatments. We have developed a gene-activated scaffold platform using a bone-mimicking collagen hydroxyapatite scaffold loaded with chitosan nanoparticles carrying genes encoding osteogenic (BMP-2) and angiogenic (VEGF) proteins. With a single treatment, protein expression by mesenchymal stem cells (MSCs) seeded onto the scaffold is sustained for up to 28 days and is functional in inducing MSC osteogenesis. The *in vivo* safety and efficacy of this gene-activated scaffold platform was demonstrated resulting in the successful transfection of host cells, abrogating the requirement for multiple procedures to isolate cells or *ex vivo* cell culture. Furthermore, the level of bone formation at the exceptionally early time-point of 28 days was comparable to that achieved following recombinant BMP-2 protein delivery after 8 weeks *in vivo*, without the adverse side effects and at a fraction of the cost. This naturally derived cell-free gene-activated scaffold thus represents a new 'off-the-shelf' product capable of accelerating bone repair in critical-sized bone defects.

1. Introduction

There remains a significant clinical challenge in the treatment of large segmental bone defects above a critical size, in particular if the vascular supply to the tissue is damaged, often leading to non-union of bone [1]. Next generation tissue engineering strategies are not only designed to mimic the native extracellular tissue but also function to induce cells to proliferate, differentiate and ultimately recapitulate the natural repair process. Within our group, we have developed a series of collagen-based scaffolds [2–4] which have shown excellent results in small [5–7] and large [8] animal models with one in particular, a collagen hydroxyapatite bone mimicking composite, currently being used in human patients following successful animal trials [9,10]. However, in large bone defects, as well as inducing bone tissue formation to fill the defect, stimulating an adequate vascular network is vital as excess matrix deposition at the periphery of the scaffold has been shown to inhibit vascularisation within the scaffold leading to cell necrosis and failure of the graft [5]. To stimulate vessel formation and accelerate bone matrix deposition, bioactive cues can be incorporated into the scaffold.

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Studying cell signalling during normal fracture repair can provide insight into the key bioactive molecules involved in bone repair.

Bone morphogenetic protein-2 (BMP-2) is a key growth factor involved in bone fracture healing, inducing cell proliferation and differentiation of progenitor cells to osteoblasts which form bone extracellular matrix [11,12]. For this reason, BMP-2 is the growth factor most commonly used to stimulate bone formation clinically [13–15]. Angiogenic factors including vascular endothelial growth factor (VEGF) are also highly up-regulated and play a vital role throughout the repair process; as well as promoting endothelial and progenitor cell migration and proliferation and subsequent vessel formation, hypertrophic chondrocytes and osteoblasts also produce VEGF, revealing the intimate relationship between VEGF and osteogenesis [16–20]. Indeed, the importance of VEGF in fracture repair has been highlighted in studies where VEGF has been inhibited, delaying callus conversion to bone [21]. Together, both BMP-2 and VEGF work in synergy with each other during bone formation and repair in a phenomenon known as osteogenic-angiogenic coupling [22–26]. We hypothesise that a scaffold that can mimic osteogenic-angiogenic coupling might enhance neovascularisation, progenitor cell recruitment and differentiation and thus accelerate the bone repair process.

Delivery of growth factors such as BMP-2 and VEGF also remains a clinical challenge due to the extremely short half-life of growth factors *in vivo*, especially in the harsh environment directly following a bone fracture [27]. Because of their instability, these growth factors are normally administered in high doses to exert a therapeutic effect which not only increases the cost, but can be cytotoxic and lead to ectopic bone formation among other side effects [28,29]. As an alternative strategy, gene therapy might provide an opportunity to control the release of growth factor(s) and avoid the requirement for repeat dosing as the cells can be genetically manipulated to over-express the growth factor(s) of choice. In this way, a more physiologically appropriate amount of growth factor might be produced by the cells for a controllable and sustained period, significantly reducing off-target side effects and cytotoxicity. However, delivery of genes *in vivo* remains a key challenge as systemic administration significantly reduces cell targeting specificity and increases the risk of off-target side effects [31], while *ex vivo* transduction of cells requires multiple procedures for harvesting and re-implantation and cells can be adversely affected by *ex vivo* culture.

Previous work within our group has led to the development of a gene-activated scaffold platform comprised of biocompatible and biodegradable chitosan (CS) nanoparticles carrying plasmid DNA (pDNA), loaded into a collagen-based scaffold which acts both as a delivery device for the CS-pDNA nanoparticles as well as filling the defect and allowing for host cell infiltration [32]. The transfection efficiency of the CS-pDNA nanoparticles was approximately 45% in mesenchymal stem cells (MSCs) and the nanoparticles did not have a cytotoxic effect. The gene-activated scaffold platform was capable of inducing transient over-expression of reporter genes for up to 28 days post-cell seeding, avoiding the risks associated with viral and synthetic gene delivery vectors and making this system ideal for tissue engineering [33,34]. As osteogenic-angiogenic coupling is crucial in bone repair, the first aim of this study was to use the gene-activated scaffold platform to mimic normal fracture repair by delivering osteogenic (BMP-2) and angiogenic (VEGF) genes and assess its functionality *in vitro*. To see the true efficacy of the genes, the initial phase of this study was performed on a collagen scaffold, however, when the optimal gene/gene combination was chosen, the collagen hydroxyapatite bone-mimicking scaffold replaced the collagen scaffold due to its superior osteoinductive capacity. Secondly, to avoid the requirement for multiple procedures or *ex vivo* culture of cells prior to implantation, assessing the functionality of the gene-activated scaffold to transfect host cells when implanted cell-free *in vivo* was critical. The third part of the study explored the ability of the gene-activated scaffold, with chitosan nanoparticles carrying both the BMP-2 and VEGF genes, to accelerate repair of critical-sized calvarial defects *in vivo*.

2. Materials and methods

2.1. Effect of BMP-2 and VEGF protein production by transfected MSCs on osteogenesis in 2D monolayer culture

2.1.1. Plasmid propagation

Plasmid DNA encoding green fluorescent protein (pGFP) purchased from Lonza, bone morphogenetic protein-2 (pBMP-2), kindly donated by Prof. Kazihusa Bessho, Kyoto University, Japan and, vascular endothelial growth factor (pVEGF) purchased from Genecopaeia (USA), all under the control of the cytomegalovirus promoter, were propagated by transforming One Shot® TOP10 Chemically Competent *E. coli* bacterial cells according to the manufacturer's protocol. pDNA was purified and collected following amplification using an Endotoxin-free Maxi-prep kit (Qiagen, UK). Plasmid was dissolved in TE Buffer at a concentration of 0.5 mg/mL and stored at −20 °C.

2.1.2. Chitosan-pDNA nanoparticle formulation

Chitosan (Mw 7.3 kDa; DD >97%) was supplied by Novamatrix, FMC Biopolymer, Norway. Nanoparticles were formulated by electrostatic interaction between cationic chitosan and anionic pDNA. Nanoparticles were allowed to equilibrate for 30 min at room temperature before use. The ratio of chitosan to pDNA (N/P ratio) was 20 and the pDNA loading dose was 2 μg as optimised previously [32].

2.1.3. Mesenchymal stem cell culture

Mesenchymal stem cells (MSCs), isolated from rat bone marrow (Caltag Medsystems, UK) were expanded in Dulbecco's Modified Eagles Medium supplemented with 2% penicillin/streptomycin, 10% FBS (Labtech, UK), 1% glutamax (Biosciences, Ireland) and 1% non-essential amino acids (Biosciences, Ireland). Cells were passaged at 70–90% confluency and expanded to passage 5 for all experiments.

2.1.4. Mesenchymal stem cell transfection in 2D culture

MSCs were seeded at a density of 5×10^4 cells per well in 6 well adherent plates (Corning, Costar, Ireland) 24 h prior to transfection. Media was removed from cells 1 h prior to transfection and cells were washed in PBS and 1 mL of OptiMEM (Gibco, Ireland) was added. Nanoparticles were made as described in Section 2.1.2 and, following complexation, OptiMEM was added in a 1:1 ratio to the nanoparticle mixture to produce the transfection medium of which 500 μL was added to each well. After 5 h, transfection media was removed, cells were washed twice in PBS and growth media was replenished.

2.1.5. Enzyme-linked immunosorbent assay (ELISA) for BMP-2 and VEGF quantification post transfection

ELISAs (R&D Systems, UK) were used to quantify the levels of BMP-2 and VEGF expressed by cells transfected with each gene as well as the combination of both genes. The cell culture supernatant was collected and analysed at days 3, 7, 10 and 14. Assays were carried out according to the manufacturer's instructions and the absorbance of each sample was read at 450 nm using a Varioskan Flash multimode plate reader (Fisher Scientific, Ireland) and the quantity of BMP-2 and/or VEGF protein present was deduced by calculating against a standard curve.