

GENE DELIVERY OF TGF- β 3 AND BMP2 IN A MSC LADEN ALGINATE HYDROGEL FOR ARTICULAR CARTILAGE AND ENDOCHONDRAL BONE TISSUE ENGINEERING

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ABSTRACT

Incorporating therapeutic genes into 3D biomaterials is a promising strategy for enhancing tissue regeneration. Alginate hydrogels have been extensively investigated for cartilage and bone tissue engineering, including as carriers of transfected cells to sites of injury, making them an ideal gene delivery platform for cartilage and osteochondral tissue engineering. The objective of this study was to develop gene-activated alginate hydrogels capable of supporting nanohydroxyapatite (nHA)-mediated non-viral gene transfer to control the phenotype of mesenchymal stem cells (MSCs) for either cartilage or endochondral bone tissue engineering. To produce these gene-activated constructs, MSCs and nHA complexed with plasmid DNA (pDNA) encoding for TGF- β 3 (pTGF- β 3), BMP2 (pBMP2), or a combination of both (pTGF- β 3/pBMP2), were encapsulated into alginate hydrogels. Initial analysis using reporter genes showed effective gene delivery and sustained overexpression of the transgenes was achieved. Confocal microscopy demonstrated that complexing the plasmid with nHA prior to hydrogel encapsulation led to transport of the plasmid into the nucleus of MSCs, which did not happen with naked pDNA. Gene delivery of TGF- β 3 and BMP2 and subsequent cell-mediated expression of these therapeutic genes resulted in a significant increase in sGAG and collagen production, particularly in the pTGF- β 3/pBMP2 co-delivery group in comparison to the delivery of either pTGF- β 3 or pBMP2 in isolation. In addition, stronger staining for collagen type II deposition was observed in the pTGF- β 3/pBMP2 co-delivery group. In contrast, greater levels of calcium deposition were observed in the pTGF- β 3 and pBMP2 only groups compared to co-delivery, with strong staining for collagen type X deposition, suggesting these constructs were supporting MSC hypertrophy and progression along an endochondral pathway. Together these results suggest that the developed gene-activated alginate hydrogels were able to support transfection of encapsulated

MSCs and directed their phenotype towards either a chondrogenic or osteogenic phenotype depending on whether TGF- β 3 and BMP2 were delivered in combination or in isolation.

1. INTRODUCTION

Adult mesenchymal stem cells (MSCs) are a promising cell source for cell-based cartilage tissue engineering strategies due to their capacity to differentiate into cells of the chondrogenic lineage¹. In comparison to chondrocytes, MSCs can be obtained in high numbers from a variety of adult tissues *via* minimally invasive procedures^{1,2} and can be expanded in culture without losing their chondrogenic differentiation potential, even when isolated from elderly or diseased donors^{3,4}. A major concern with the use of MSCs for articular cartilage regeneration is their inherent tendency to progress towards hypertrophy and endochondral ossification or to undergo fibrous dedifferentiation^{2,5}. However, this inherent potential of chondrogenically primed MSCs to become hypertrophic can be leveraged for endochondral bone tissue engineering, which aims to recapitulate embryonic skeletal development in order to engineer fully functional bone tissue⁶⁻¹⁰. Ultimately, it is important to be able to control the MSC phenotype towards a particular target application (e.g. stable articular cartilage or bone). One way to achieve this is through the use of gene or growth factor delivery in combination with biomaterials for tissue repair.

Recombinant growth factor administration has been widely investigated for tissue engineering, however the delivery of such factors *in vivo* for therapeutic effect is hampered by issues such as their short half-life, transient action, and side effects associated with the need for delivery of high concentrations of protein to elicit a therapeutic outcome¹¹. Non-viral gene therapy may provide a more physiological, durable and cost-effective alternative^{12,13}. Expression of the gene product also guarantees authentic post-translational modifications reducing possible immunogenicity and increasing biological activity in comparison to pre-synthesised recombinant proteins¹⁴. Additionally, gene therapy allows for a simpler way of simultaneous and sequential delivery of cell-mediated growth and transcription factors that could enhance the multifactorial process of articular cartilage regeneration. In tissue engineering applications, gene therapeutics can be combined with biomaterials for a prolonged, sustained and localized *in situ* delivery of a protein of interest. This approach may overcome the limitations associated with 2D transfection^{15,16} and direct injection which are not ideal for targeting a specific tissue or cell type¹⁷. Hydrogels are extensively used in tissue engineering to provide cells with a three-dimensional (3D) environment similar to native extracellular matrix (ECM). Hydrogels offer many advantages over pre-fabricated porous scaffolds; they are typically more compatible with minimally invasive delivery strategies, permit efficient oxygen and nutrient transport, facilitate homogeneous cell distribution, and can be used in the treatment of irregular shaped defects. However, to date, only

a small number of studies have explored the use hydrogels as gene delivery platforms or gene activated matrices (GAMs) for articular cartilage tissue engineering^{18,19}.

Alginate is a naturally occurring anionic polymer that is ideally suited to cartilage and bone tissue engineering due to its biocompatibility, tailorable degradation kinetics and low toxicity²⁰. Encapsulation of MSCs in alginate hydrogels has demonstrated potential for both cartilage and bone regeneration *in vitro* and *in vivo*²¹⁻²⁵. Alginate hydrogels have also been used as a 3D gene delivery platform for bone tissue engineering applications²⁶⁻²⁹, and to support transfected chondrocytes for articular cartilage regeneration^{18,19,30}. This hydrogel therefore has broad potential applications in orthopedic medicine if appropriate strategies can be developed to control therapeutic gene delivery and hence cell fate within such constructs. The overall objective of this study was thus to develop and characterise a novel nanohydroxyapatite (nHA)-mediated non-viral gene-activated alginate hydrogel capable of directing MSC fate by delivering the therapeutic genes TGF- β 3 and/or BMP-2. It was hypothesized that chondrogenesis and the progression towards hypertrophy and endochondral ossification within such an alginate construct can be either suppressed or enhanced by the delivery of specific genes to encapsulated MSCs.

2. MATERIALS AND METHODS

2.1. Experimental design

In order to achieve the objectives of this study, a series of different experiments were performed. Firstly, nHA transfection efficiency and its effects on cell viability were assessed in 2D using PEI as a control. Secondly, nHA-gene-activated alginate hydrogels were produced and reporter genes encoding for green fluorescent protein (pGFP) and luciferase (pLuc) were used to determine the capacity of these gels to transfect MSCs over time and to explore their potential cytotoxicity. Additionally, tracking of nHA-pGFP complex uptake in the gene-activated alginate hydrogels was performed through pDNA labelling and cell fluorescent staining using confocal microscopy. Thirdly, the effects of the delivery of the therapeutic genes TGF- β 3 and BMP2 in isolation or in combination on MSCs encapsulated within these hydrogels were assessed. Specific protein expression was confirmed using ELISA and differentiation of the MSCs was assessed through biochemical, histological and immunohistochemical analysis of the secreted extracellular matrix components. Mechanical testing of the gene activated hydrogels was also conducted.

2.2. Isolation and expansion of bone marrow derived MSCs

Bone marrow-derived stem cells (BMSCs) were isolated from the femora of porcine donors (3-4 months, >50 kg) within 3 hours of sacrifice according to a modified method developed for human MSCs³¹. Mononuclear cells were plated at a seeding density of 5×10^3 cells/cm² in standard culture media containing high-glucose Dulbecco's modified eagles medium (4.5 mg/mL D-Glucose, 200 mM L-Glutamine; hgDMEM) supplemented with 10% fetal bovine serum (FBS),

penicillin (100 U/mL)–streptomycin (100 g/mL) (all GIBCO, Biosciences, Dublin, Ireland), and expanded to passage two in a humidified atmosphere at 37 °C, 5% CO₂ and 20% pO₂.

2.3. Plasmid propagation

Four different plasmids were used in the current study, two plasmids encoding for the reporter genes luciferase (pGaussia- Luciferase, New England Biolabs, Massachusetts, USA) and green fluorescent protein (pGFP) (Amara, Lonza, Cologne AG, Germany), and another two encoding for the therapeutic genes bone morphogenic protein 2 (BMP2) (the BMP2 plasmid was a kind donation from Prof. Kazihusa Bessho, Kyoto University, Japan) and transforming growth factor beta 3 (TGF-β3) (InvivoGen, California, USA). Plasmid amplification was performed by transforming One Shot® TOP10 Chemically Competent E. coli bacterial cells (Biosciences, Ireland) according to the manufacturer's protocol. The transformed cells were cultured on LB plates with 50 mg/L kanamycin (Sigma-Aldrich, Ireland) as the selective antibiotic for pGFP, and 100 mg/L of ampicillin (Sigma-Aldrich, Ireland) as the selective antibiotic for pLuc, pTGF-β3 and pBMP2 competent bacteria. Bacterial colonies were harvested and inoculated in LB broth (Sigma-Aldrich, Ireland) overnight for further amplification. The harvested bacterial cells were then lysed and the respective pDNA samples were collected using a MaxiPrep kit (Qiagen, Ireland). Nucleic acid content was analysed using NanoDrop 1000 spectroscopy, taking the 260/280 ratio and 230 nm measurement to determine the ng/μl measurement. Plasmids in this study were used at a concentration of 0.5 μg of plasmid in 1 μl of Tris-EDTA (TE) buffer.

2.4. Nanohydroxyapatite (nHA)–plasmid DNA (pDNA) complex formation and monolayer transfection

The synthesis of the nHA nanoparticles was performed as previously described³². Briefly, a solution of 12 mM sodium phosphate, containing 0.017% Darvan 821A (RT Vandervilt, Norwalk, CT), was added to an equal volume of a 20 mM calcium chloride solution and filtered through a 0.2 μm filter. The nHA-pDNA transfection mix consisted of 150 μl (monolayer transfections) or 50 μl (gene-activated alginate hydrogels) of the nHA solution added to the pDNA (specific amounts detailed below) combined with 0.25 M of CaCl₂. Monolayer nHA-pGFP and nHA-pLuc transfections were performed adding the 2 μg of nHA-pGFP or nHA-pLuc complexes suspended in 500 μl standard culture media to BMSCs seeded in 6 well plates at a cell density of 5 x 10⁴ cells/well. Residual complexes were removed after 4 hours of incubation and replaced with fresh culture media.

2.5. Polyethylenimine (PEI)-pDNA complex formation and monolayer transfection

PEI was used as a positive control for the determination of the transfection efficiency of the MSCs in monolayer. Branched 25kDa PEI (Sigma-Aldrich, Ireland) was condensed with pDNA encoding for GFP in an N/P ratio = 7. PEI-pGFP monolayer transfections were performed adding

2 µg of the PEI-pGFP complexes suspended in 500 µl of Opti-MEM Reduced Serum Media (Life Technologies, Dun Laoghaire, Ireland) to BMSCs seeded in 6 well plates at a cell density of 5×10^4 cells/well. PEI-pGFP complexes were removed after 4 hours of incubation and new standard culture media was added.

2.6. Assessment of MSC monolayer transfection efficiency

Analysis of monolayer transfection efficiency was conducted by determining the percentage of green fluorescent cells at 3 and 7 days post-transfection in relation to the whole population through cell sorting using a BD Accuri™ C6 flow cytometer.

2.7. Assessment of metabolic activity and cell viability

Cell metabolic activity was evaluated using AlamarBlue™ (BioSciences, Dun Laoghaire, Ireland), a non-endpoint, non-toxic assay, after 3 and 7 days of nHA and PEI-mediated gene delivery in monolayer, and after 1 and 7 days in the 3D gene-activated alginate hydrogel system. 10% AlamarBlue™ in 1 ml of standard culture media was used for the assay. All samples were incubated for 4 hours at 37 °C. After the incubation time, 200 µl of the supernatant was plated in triplicate into a 96-well plate, absorbance was read at 570 nm and 600 nm and its reduction was translated to cell activity relative to the untransfected control.

For nHA-pLuc transfected groups, cell viability was assessed using LIVE/DEAD® Viability/Cytotoxicity Assay Kit (Invitrogen, Bio-science, Ireland). Images were taken by confocal microscopy using an Olympus FV-1000 Point-Scanning Confocal Microscope (South-on-Sea, UK) at 488 and 543 nm channels and analysed using FV10-ASW 2.0 Viewer software, and cell viability was calculated using ImageJ software.

DNA analysis was performed by digesting the samples with papain (125 µg/ml, pH = 6.5) in 0.1 M sodium acetate, 5 mM L-cysteine HCl, and 0.05 M EDTA (Sigma-Aldrich, Ireland) at 60 °C under constant rotation for 18 hours. DNA content was quantified using the Hoescht 33258 dye-based DNA QF kit and standard curve (Sigma-Aldrich, Ireland).

2.8. Production of the control and gene-activated alginate hydrogels

Expanded BMSCs were trypsinised and counted using trypan blue exclusion staining. For the untransfected control groups they were encapsulated in 2% alginate yielding a final concentration of 1×10^7 cells/ml of alginate (5×10^5 cells per hydrogel). For the gene-activated alginate hydrogels, the trypsinised BMSCs were incubated with different concentrations of pDNA complexed to nHA nanoparticles for 60 minutes prior to alginate encapsulation. In the case of pGFP-gene-activated hydrogels, 2 µg of pDNA per hydrogel were used. For pLUC-gene activated hydrogels, 2 µg, 5 µg or 10 µg per hydrogel were used. For therapeutic gene delivery, 2 µg of pBMP2 (pBMP2 group), 2 µg of pTGF-β3 (pTGF-β3 group) and a combination of 2 µg of pBMP2 and 2 µg of pTGF-β3 (pTGF-β3/pBMP2 group) per hydrogel were used. After incubation, the

BMSCs and the pDNA-nHA complexes were encapsulated in 2% alginate for a final concentration of 1×10^7 cells/ml of alginate.

Alginate/cell suspensions were pipetted into 3% agarose/100 mM CaCl₂ cylindrical moulds and the gels were allowed to ionically crosslink within these moulds at room temperature for 15 minutes to form cylindrical constructs (Ø5 x H3 mm).

2.9. Characterization of nHA-pDNA complex uptake and internalization in the gene-activated alginate hydrogels

Cellular uptake and internalization of labelled pDNA on its own and complexed to nHA nanoparticles were assessed through confocal microscopy using an Olympus FV-1000 Point-Scanning Confocal Microscope (South-on-Sea, UK). Actin cellular cytoskeleton staining with Alexa488 Phalloidin (Invitrogen, Ireland) and nuclear staining with diamidino-2-phenylindole (DAPI, Invitrogen, Ireland) and cyanine 3 (Cy3, LifeTechnologies, Ireland) labelling of pDNA encoding for luciferase were performed. Constructs were fixed in 4% paraformaldehyde at 4, 24 and 72 hours post-transfection and were subsequently imaged using confocal microscopy.

2.10. Hydrogel culture

For assessment of reporter gene expression, alginate hydrogels were cultured in standard culture medium in 24 well plates with 1 gel per well in a humidified atmosphere at 37 °C, 5% CO₂ and 20% O₂. Each construct was maintained in 1.5 mL of medium with complete media changes performed twice weekly.

For differentiation of MSCs, after 1 day in standard culture media, the alginate hydrogels were cultured in a chemically defined medium (CDM) consisting of DMEM GlutaMAX™ supplemented with penicillin (100 U/ml)-streptomycin (100 µg/ml) (both Gibco, Biosciences, Ireland), 100 µg/ml sodium pyruvate, 40 µg/ml L-proline, 50 µg/ml L-ascorbic acid-2-phosphate, 1.5 mg/ml bovine serum albumin (BSA), $1 \times$ insulin–transferrin–selenium (ITS), 100 nM dexamethasone (all from Sigma-Aldrich, Ireland), in 24 well plates with 1 gel per well in a humidified atmosphere at 37 °C, and hypoxic conditions of 5% O₂ and 5% CO₂ for 28 days. Each construct was maintained in 1.5 mL of medium with complete medium changes performed twice weekly.

2.11. Enzyme-linked Immunosorbent Assay (ELISA) for BMP2 and TGF-β3 quantification post-transfection

The levels of BMP2 and TGF-β3 in the culture medium expressed by transfected MSCs encapsulated in the gene-activated alginate hydrogels (n = 4) were quantified using ELISAs (Koma Biotech, Korea). The cell culture supernatant was collected and analysed at the following time-points: day 1, day 3, day 7, day 10 and day 14. Assays were carried out according to the manufacturer's instructions and the absorbance of each sample was read at 450 nm using a plate

reader whereby the quantity of either BMP2 or TGF- β 3 protein present was deduced by calculating against a standard curve.

2.12. Quantitative biochemical analysis

Samples were digested with papain (125 μ g/ml, pH = 6.5) in 0.1 M sodium acetate, 5 nM L-cysteine HCl, and 0.05 M EDTA (Sigma-Aldrich, Ireland) at 60 $^{\circ}$ C under constant rotation for 18 hours. DNA content was quantified using the Hoescht 33258 dye-based DNA QF kit (Sigma-Aldrich, Ireland). Proteoglycan content was estimated by quantifying the amount of sulphated glycosaminoglycan (sGAG) in the gels using the dimethylmethylene blue (BDMMB) dye-binding assay (Blyscan, Biocolor Ltd, Northern Ireland), with a chondroitin sulphate standard. Total collagen content was determined by measuring the hydroxyproline content. Samples were hydrolysed at 110 $^{\circ}$ C for 18 hours in concentrated hydrochloric acid (HCl) 38%, allowed to dry and analysed using a chloramine-T assay³³ using a hydroxyproline-to-collagen ratio of 1 : 7.69³⁴. Calcium content was determined using a Sentinel Calcium Kit (Alpha Laboratories Ltd, UK) after digestion in 1 M HCl at 110 $^{\circ}$ C for 36 hours. Four constructs per group were analysed for each biochemical assay.

2.13. Histological and immunohistochemical analysis

Constructs were fixed in 4% paraformaldehyde, dehydrated in a graded series of ethanol baths, embedded in paraffin wax, sectioned at 8 μ m and affixed to microscope slides. The sections were stained with 1% alizarin red (Sigma-Aldrich, Ireland) to assess calcium accumulation, aldehyde fuschin/alcian blue (Sigma-Aldrich, Ireland) to assess sGAG content, and picrosirius red (Sigma-Aldrich, Ireland) to assess collagen production after 28 days of *in vitro* culture. Collagen types II and X were evaluated using a standard immunohistochemical technique as described previously⁹. Negative and positive controls of porcine ligament, cartilage and growth plate were included for each immunohistochemical analysis.

2.14. Mechanical Testing

Constructs were mechanically tested (n=3) in unconfined compression using a standard material testing machine with a 5 N load cell (Zwick Roell Z005, Herefordshire, UK). Briefly, constructs were kept hydrated through immersion in Dulbecco's modified eagles medium (GIBCO, Biosciences, Dublin, Ireland) bath maintained at room temperature. A pre-load of 0.01 N was applied to ensure that the construct surface was in direct contact with the impermeable loading platens. Stress relaxation tests were performed consisting of a ramp displacement of 1 m/s up to 10% strain, which was maintained until equilibrium was reached (~30 min).

2.15. Statistical analysis

Statistical analyses were performed using GraphPad Prism (version 5) software with 3-4 samples analysed for each experimental group. Pairwise comparisons between means of different groups were performed using a Student t-test. Two-way ANOVA was used for analysis of variance with Tukey's post-hoc test to compare between groups. Numerical and graphical results are displayed as mean \pm standard deviation. Significance was accepted at a level of $p < 0.05$.

3. RESULTS

3.1. nHA can be used to effectively transfect MSCs

PEI and nHA demonstrated a comparable capacity to transfect bone marrow derived MSCs (BMSCs) in monolayer culture, with approximately 20% of cells GFP positive at day 3 in both groups. At day 7 the percentage of GFP positive cells significantly decreased in the nHA-pGFP group, but stayed constant in the PEI group (Fig.1.A). Assessment of cell viability using alamar blue showed significantly higher cell metabolic activity in the untransfected control and the nHA-pGFP group in comparison to the PEI transfected cells at day 3 (Fig.1.B), suggesting that PEI has a negative effect on the metabolic activity of MSCs. Increased metabolic activity was observed in the nHA-pGFP group showed at day 7 compared to the control and PEI-pGFP constructs (Fig.1.B).

3.2. Alginate hydrogels are able to support nHA-mediated gene delivery and sustained expression of the transgene over time without negatively impacting cell viability

Quantification of luciferase expression confirmed effective gene delivery to MSCs in the gene-activated alginate hydrogels containing nHA complexed with different concentrations of pLuc, with sustained expression of the transgene over 14 days (Fig.2.A). The luciferase expression peaked at day 7 in all gene-activated groups (Fig.2.A), in comparison to 2D transfection in which the luciferase expression peaked at day 3, and was not significantly different to the untransfected control by day 7 (Sup. Fig.1). The use of 2 μ g of pLuc per gel appeared to be the optimal concentration of pDNA, as evidenced by significantly higher levels of luciferase expression in comparison to 5 μ g and 10 μ g of pLuc at specific time points (Fig.2.A). Importantly, luciferase expression was also sustained over 14 days within the 2 μ g of pLuc per hydrogel group.

Fluorescent inverted microscope images of the gene-activated alginate hydrogels containing MSCs and nHA-pGFP complexes revealed the presence of green fluorescent cells inside the hydrogels, indicating effective transfection and GFP production over 23 days (Fig.2.B). Fluorescent staining of the cellular actin cytoskeleton and the nucleus of the encapsulated MSCs, and pDNA encoding for luciferase, showed naked pLuc and nHA-pLuc uptake over 72 hours (Fig.2.C). pDNA could be observed inside the cell cytoplasm and nucleus of the MSCs in the

nHA-pLuc group at 4, 24 and 72 hours after encapsulation in alginate. In contrast, the pDNA was completely degraded after 72 hours in the naked pLuc group and it could not be observed inside the cellular compartment or around the cell membrane (Fig.2.C). Additionally, imaging of the nHA-pLuc group suggested the presence of stable nHA-pDNA complexes inside and outside the cell over 72 hours post transfection (Fig.2.C).

No significant differences were observed between the DNA content and the cell metabolic activity of the nHA-pLuc gene-activated hydrogels and the untransfected controls at day 1 and 7 (Fig.3.A and B), suggesting that cell viability was unaffected by the incorporation of the complex into the alginate hydrogels. Confocal imaging of live and dead cells revealed a homogeneous distribution of the cells throughout each gel, with no obvious differences in cell viability between the groups (Fig.3.C and D).

3.3. Addition of therapeutic genes into 3D alginate hydrogels results in effective delivery and sustained expression of the transgene

Successful expression of therapeutic proteins was achieved using nHA-mediated transfection of MSCs within the alginate hydrogels. This resulted in increased levels of TGF- β 3 (Fig.4.A) and BMP2 (Fig.4.B) in the culture media of the pTGF- β 3, pBMP2 and pTGF- β 3/pBMP2 co-delivery groups (Fig.4). The peak of expression was observed at day 7 for TGF- β 3 and at day 3 for BMP2, high reduction in the expression of the gene products was observed at day14. These results verify effective MSC transfection with pTGF- β 3 and pBMP2 within the gene-activated hydrogels and demonstrate sustained expression of the genes of interest. A similar trend and level of expression was observed for each protein, independent of whether the pDNA had been delivered in isolation or in combination with another pDNA, implying that co-delivery of two plasmids did not hinder the expression of either gene.

3.4. Co-delivery of TGF- β 3 and BMP2 genes enhances chondrogenesis and suppresses hypertrophy and calcification compared to delivery of either gene in isolation

pTGF- β 3/pBMP2 co-delivery led to significantly higher levels of sGAG and collagen accumulation compared to all other groups after 28 days of culture (Fig.5.A and C). Collagen accumulation in the pTGF- β 3 group was significantly higher than in pBMP2 and nHA-alone groups (Fig.5.C). Similar trends were found when the sGAG and collagen content were normalized to the DNA levels (Fig.5.B and D). In contrast, significantly higher levels of calcium deposition were observed in both the pTGF- β 3 and pBMP2 only groups in comparison to the co-delivery of both genes (Fig.5.E and F). At the levels of nHA used within this study, no evidence of osteoinductivity or calcification was observed in a nHA alone control group (Fig.5.E and F).

The equilibrium modulus (Fig.5.H) of the pTGF- β 3/pBMP2 co-delivery group was significantly higher than the nHA-only control after 28 days of *in vitro* culture, with a similar trend observed for the Young's modulus (Fig.5.G).

Histological evaluation of the hydrogels after 28 days in culture demonstrated higher pericellular sGAG and type II collagen deposition in the pTGF- β 3/pBMP2 co-delivery constructs compared to all other groups (Fig. 6). Greater calcification (Fig.6.G and H) was observed in the pBMP2 and pTGF- β 3 delivery only groups. Aldehyde fuschin/alcian blue and picrosirius red staining revealed higher sGAG (Fig.6.A, B and C) and collagen (Fig.6.D, E and F) accumulation in the pTGF- β 3/pBMP2 co-delivery group, confirming the results of the biochemical analysis. Minimal immunostaining for collagen type X, a marker of hypertrophy, was seen in the pTGF- β 3/pBMP2 co-delivery group compared to the pTGF- β 3 and pBMP2 only constructs (Fig.6.M, N and O), with the most intense staining in the pBMP2 group. These results suggest enhancement of chondrogenesis in the co-delivery group, and the promotion of hypertrophy and calcification when TGF- β 3 and BMP2 genes are delivered in isolation using nHA as a delivery vector. Confocal imaging of live and dead cells confirmed the presence of living cells in all the groups after 28 days of *in vitro* culture (Fig.6.P, Q and R).

4. DISCUSSION

The overall goal of the present study was to develop a novel nHA-mediated gene-activated alginate hydrogel capable of supporting sustained delivery of pDNA encoding for therapeutically relevant factors to MSCs in order to control their differentiation for either articular cartilage or endochondral bone tissue engineering. Luciferase expression analysis, fluorescent imaging and cell viability assessment confirmed effective gene delivery to MSCs encapsulated within the gene-activated hydrogels without any toxic effects. Therapeutic gene delivery of TGF- β 3 and BMP2 and subsequent expression of the transgenes promoted sGAG and collagen type II production when the two genes were delivered in combination, or calcification and collagen type X deposition when these genes were delivered in isolation. Taken together, these results indicate that the nHA-gene-activated hydrogels are able to efficiently sustain non-viral transfection and expression of the genes of interest over a temporary timeframe, providing relevant concentrations of proteins which modulated the phenotype of MSCs towards either cartilage or down an osteogenic/endochondral pathway depending on whether TGF- β 3 and BMP2 were delivered in combination or in isolation.

In-house synthesized hydroxyapatite (HA) nanoparticles showed transfection efficiencies similar to PEI, but without the cytotoxicity associated with this cationic polymer. HA nanoparticles have been investigated as delivery vectors for diverse biomedical applications including growth factors³⁵, anticancer drugs³⁶, enzymes and antibodies³⁷, and nucleic acids such

as plasmid DNA^{38,39} and small interfering RNA (siRNA)⁴⁰. In terms of gene delivery, HA nanoparticles have a high binding affinity for pDNA due to the interactions between the calcium ions in the apatite and the negatively charged phosphate groups of DNA⁴¹. Previous studies have reported MSC transfection efficiency to be higher using nHA than commercial calcium phosphate (CaP) kits, and to offer better cell viability than Lipofectamine 2000³⁹, the most referenced transfection reagent. Additionally, nHA-mediated gene delivery within collagen scaffolds have shown greater therapeutic benefits for bone regeneration *in vivo* in comparison to polyethyleneimine (PEI), which is often considered the gold standard for cationic gene delivery⁴². **Although relatively low levels of nHA-mediated transfection efficiency were reported in this study in comparison to viral vectors such as recombinant adeno-associated viruses (rAAV)^{43,44} or physical cell stimulation methods such as electroporation⁴⁵, the use of non-viral particles for gene delivery is a promising option for their application in tissue engineering approaches due to their low immunogenicity, transient effect and the ease of incorporation to 3D matrices for a sustained and localised delivery of the gene of interest.**

In this study, these nHA nanoparticles were incorporated into alginate hydrogels in order to facilitate and sustain gene delivery to MSCs over time. This resulted in effective transfection with pDNA encoding for Luc and GFP, and subsequent expression of the transgenes over 23 days as confirmed by fluorescent imaging and Luc analysis. Luc expression peaked at day 7 in the gene-activated hydrogels, suggesting a sustained uptake of the nHA-pLuc complexes over time, in comparison to the transfection with pLuc or pGFP in 2D monolayer culture, where expression peaked at day 3 and showed a significant reduction by day 7. Different pLuc concentrations (2 µg, 5 µg and 10 µg/gel) were also assessed. 2 µg/gel was found to be the optimal in terms of gene expression, with significantly higher Luc expression at every time point, which may be due to the formation of larger complexes with increasing concentrations of pDNA preventing cellular uptake⁴⁶. Cytotoxicity analysis of the system showed similar levels of DNA content, metabolic activity and percentage of living cells across the gene-activated groups and the untransfected controls, confirming the monolayer results and previous studies that highlighted the non-toxic effects of these nanoparticles³⁹, making them ideal for many tissue engineering applications.

In order to track the pDNA uptake in the gene-activated hydrogels and to better understand the efficacy of the nHA nanoparticles in the system, the internalization of pDNA was monitored through fluorescent staining at 4, 24 and 72 hours after alginate encapsulation of the nHA-pDNA complexes and MSCs. Confocal imaging of the fluorescent labelled pDNA, cellular cytoskeleton and cellular nucleus showed that the combination of nHA nanoparticles and pDNA resulted in formation of pDNA complexes, sustained cellular internalization, and protection of the internalized pDNA overtime inside the alginate hydrogels. In contrast, in the absence of the nHA

nanoparticles, the naked pDNA was not able to be internalized by the MSCs and it was not observed in the cells or their periphery after 72 hours, suggesting degradation of the plasmid. Hydroxyapatite particles have been previously reported to protect pDNA from degradation driven by serum and nucleases such as DNase I^{47,48}, these results further demonstrate this protective action. The presence of nHA also resulted in effective gene transfection, confirming that these nanoparticles acted as a successful gene delivery vector in the developed system.

nHA nanoparticles were able to effectively deliver pDNA encoding for TGF- β 3 or BMP-2 in the 3D alginate hydrogels and sustain their expression over 14 days. Media supplementation with growth factors from the transforming growth factor (TGF) family such as transforming growth factor-beta 1 (TGF- β 1) and 3 (TGF- β 3), or bone morphogenic protein 2 (BMP2), have previously been used to direct differentiation of MSCs encapsulated in alginate hydrogels towards either a chondrogenic or an osteogenic phenotype^{9,49,50}. Although relatively low levels of the transgene expression were quantified in the media (compared to studies that directly supplement the media with the gene product), it is likely that a significant proportion of the TGF- β 3 and BMP2 proteins being produced are retained within the alginate construct, particularly within the pericellular matrix (PCM) deposited by the encapsulated cells. Such matrix components have previously been shown to bind strongly with such growth factors⁵¹. In comparison to recombinant growth factor media supplementation, this approach offers a continuous production of proteins of interest which overcomes the limitations related to the short half-life and quick degradation of proteins *in vivo* and the adverse effects associated the administration of supraphysiological amounts of growth factors¹¹.

Gene delivery of either TGF- β 3 or BMP2 in isolation showed only moderate sGAG and collagen synthesis and significantly increased calcification in comparison to the pTGF- β 3/BMP2 co-delivery group. Furthermore, the delivery of these genes in isolation enhanced the production of collagen type X, a marker for chondrocyte hypertrophy and endochondral ossification. While it is perhaps unsurprising that the delivery of BMP2 appeared to support a more osteogenic phenotype^{29,52}, the finding that pTGF- β 3 delivery supported a similar phenotype was less expected. It is likely that the combination of the osteogenic stimulus provided by the nHA, with the overexpression of pTGF- β 3 led to promotion of hypertrophic/osteogenic differentiation in the MSCs⁵³. In contrast, nHA-mediated co-delivery of pTGF- β 3 and pBMP2 resulted in suppressed calcification and collagen type X deposition, and promoted a more stable chondrogenic phenotype characterized by increased GAG and collagen type II production. Combined medium supplementation of either TGF- β 3 or TGF- β 1 with BMP2 has been shown previously to synergistically enhance chondrogenic differentiation of MSCs encapsulated in alginate beads^{49,54}. This synergistic effect on MSC chondrogenesis may be produced through modulation of the Smad and MAPK signalling pathways^{55,56} and suppression of Runx2 expression⁵⁷ resulting in the

promotion of collagen type II, cartilage oligomeric matrix protein (COMP) and aggrecan (ACAN) gene expression, sGAG synthesis, and, at the same time, decreasing the expression of bone-specific alkaline phosphatase^{49,54}. While the present study shows that the co-delivery of TGF- β 3 and BMP2 produced more stable chondrogenesis and suppressed hypertrophy, Simmons *et al.* showed that the combination of TGF- β 3 and BMP2 growth factors in peptide modified alginate laden with MSCs led to higher bone formation compared to when either growth factor was delivered in isolation²⁴. This could be due to the presence of peptide modification and/or the reduced molecular weight of the alginate used in that study⁵⁸. Other factors to be considered are the relatively low concentrations of TGF- β 3 and BMP2 produced by the MSCs in the gene-activated hydrogels, and the hypoxic conditions of the *in vitro* culture which have previously shown to suppress the hypertrophic phenotype of MSCs^{59,60}. The exact molecular mechanism by which combined delivery of TGF- β 3 and BMP2 suppressed calcification *in vitro* and promoted chondrogenesis remains unclear. Its elucidation could help to understand and modulate hypertrophy and endochondral ossification of MSCs and will be addressed in future studies.

In conclusion, the nHA-gene-activated alginate hydrogels developed in this study were capable of sustaining gene transfection of MSCs, leading to transgene expression over at least 14 days of culture. The expression of the transgenes was capable of modulating stem cell fate towards either a chondrogenic or osteogenic/endochondral phenotype, depending on whether pTGF- β 3 and pBMP2 were delivered in isolation or in combination. This is the first study to show that nHA-mediated gene delivery is capable of inducing chondrogenic differentiation of MSCs, and that alginate hydrogels can be used as gene delivery platform for both cartilage and endochondral bone tissue engineering. Altogether, these results may be of clinical importance for the treatment of osteochondral defects as this system offers control of MSC fate towards either a chondrogenic or endochondral phenotype, while avoiding the risks and drawbacks associated with recombinant protein administration.

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Figure 1. (A) Percentage of GFP positive MSCs at day 3 and 7 post-transfection with nHA nanoparticles (nHA-pGFP) and PEI (PEI-pGFP) in comparison to the untransfected control; *** denotes significance (n=4, p<0.001) in comparison to the untransfected control group, * denotes significant (n=4, p<0.05) in comparison to the untransfected control group. (B) Cell metabolic activity (% of untransfected control) after 3 and 7 days of transfection with nHA nanoparticles or PEI complexed to pGFP, * denotes significance (n=4, p<0.05) in comparison to the PEI-pGFP group at day 3 and to the control and PEI-pGFP groups at day 7.

Figure 2. (A) Luciferase expression profile of the MSCs in the gene activated hydrogels containing nHA nanoparticles complexed to different concentrations of pLuc (2 ug, 5 ug and 10 ug) over 14 days; ** denotes significance (n=4, p<0.01) in comparison to the 5 µg nHA-pLuc and the 10µg nHA-pLuc groups; *** denotes significance (n=4, p<0.001) in comparison to the 5 µg nHA-pLuc, 10 µg nHA-pLuc and untransfected control groups. (B) Fluorescent inverted microscope images of the gene-activated alginate hydrogels containing MSCs and nHA-pGFP complexes over 23 days; Scale bars: 100 µm. (C) Confocal microscopy analysis of the cellular uptake and internalization of pDNA encoding for Luciferase (naked pLuc) and complexed to the nHA nanoparticles (nHA-pLuc) by MSCs encapsulated in alginate hydrogels, at 4, 24 and 72 hours post transfection and alginate encapsulation. Cellular actin cytoskeleton was stained in green, the nucleus in blue and the pDNA in red; Scale bars: 10 µm.

Figure 3. (A) DNA quantification of the nHA-pLuc gels and the untransfected control at day 1 and 7 after encapsulation. (B) Cell metabolic activity (% of untransfected control) of the nHA-pLuc gene-activated gels (nHA-pLuc) and the untransfected control (control) at day 1 and 7 after alginate encapsulation (C) Percentage of living cells in the nHA-pLuc gene-activated gels (nHA-pLuc) and the untransfected control (control) at day 1 and 7 after alginate encapsulation. (D) Cell viability at day 1 and 7; green fluorescence indicate viable cells (calcein) and red indicates dead cells (ethidium homodimer-1). 1 refers to image in the edge of the gel and 2 to the center of the gels. Scale bars = 1 mm and 0.5 mm.

Figure 4. (A) pTGF-β3 and BMP-2 (B) protein expression in the pTGF-β3 and the pTGF-β3/pBMP2 gene-activated hydrogels over 14 days.

Figure 5. Biochemical and mechanical analysis of the constructs after 28 days of *in vitro* culture. (A) sGAG content. (B) sGAG content normalized to DNA. (C) Collagen content. (D) Collagen content normalized to DNA. (E) Calcium content. (F) Calcium content normalized to DNA. (G) Young modulus and (H) equilibrium modulus. ** (n=4, p<0.01) and *** (n=4, p<0.001) denotes significance in comparison to the rest of the groups, * (n=4, p<0.05) denotes significance in comparison to the nHA-alone group, # (n=4, p<0.05) denotes significance in comparison to the pTGF-β3/pBMP2 co-delivery group.

Figure 6. Histological examination of the gene-activated hydrogels and the controls after 28 days of *in vitro* culture. (A, B and C) sGAG histological examination through aldehyde fuschin / alcian blue staining at day 28. Scale bars = 0.5 mm and 1 mm. (D, E and F) Collagen histological examination through picrosirius red staining at day 28. Scale bars = 0.5mm and 1 mm. (G, H and I) Calcium deposition histological examination through alizarin red. Scale bars = 0.5 mm and 1 mm. Collagen type II (J, K and L) and Collagen type X (M, N and O) immunostaining at day 28. Scale bars = 0.1 mm and 1 mm. (P, Q and R) Cell viability at day 28; green fluorescence indicates viable cells, red fluorescence indicates dead cells. Scale bar = 1mm.

Supplementary Figure 1. Luciferase expression at day 3 and 7 of MSCs transfected in 2D using nHA nanoparticles complexed to 2 μ g of pLuc; * denotes significance (n=3, p<0.05) in comparison to the untransfected control.

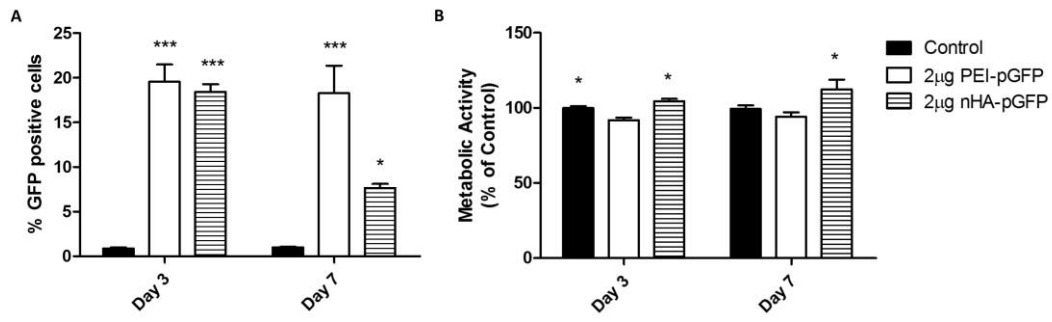


Fig.1.

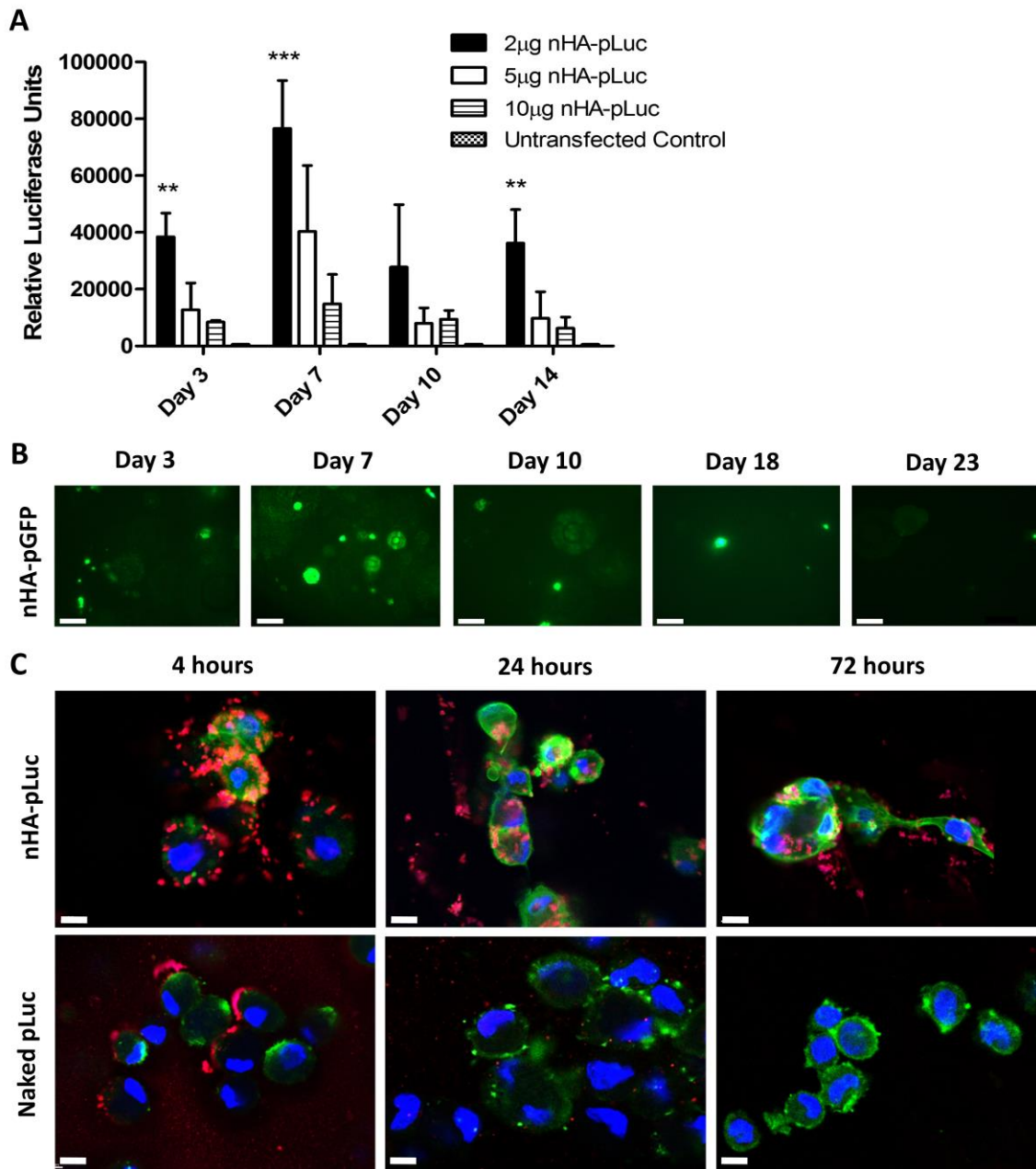


Fig.2.

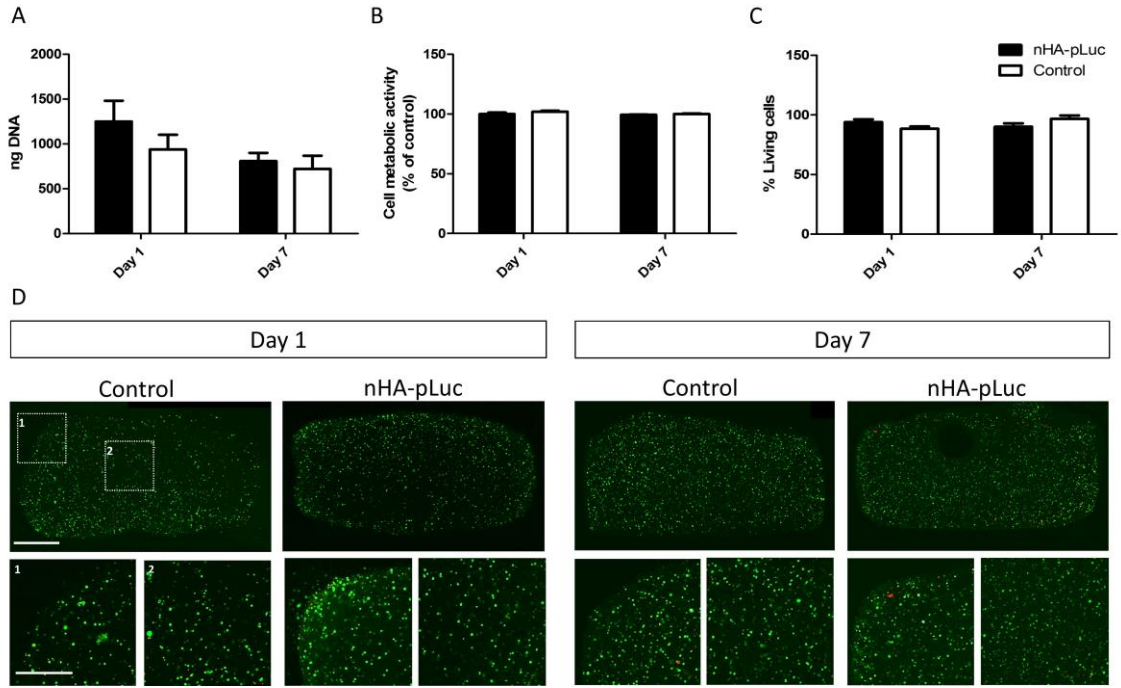


Fig.3.

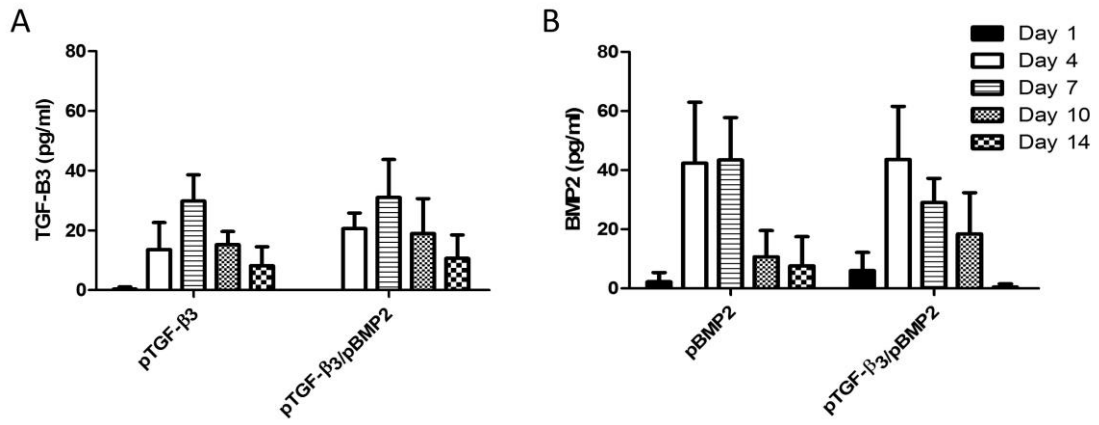


Fig.4.

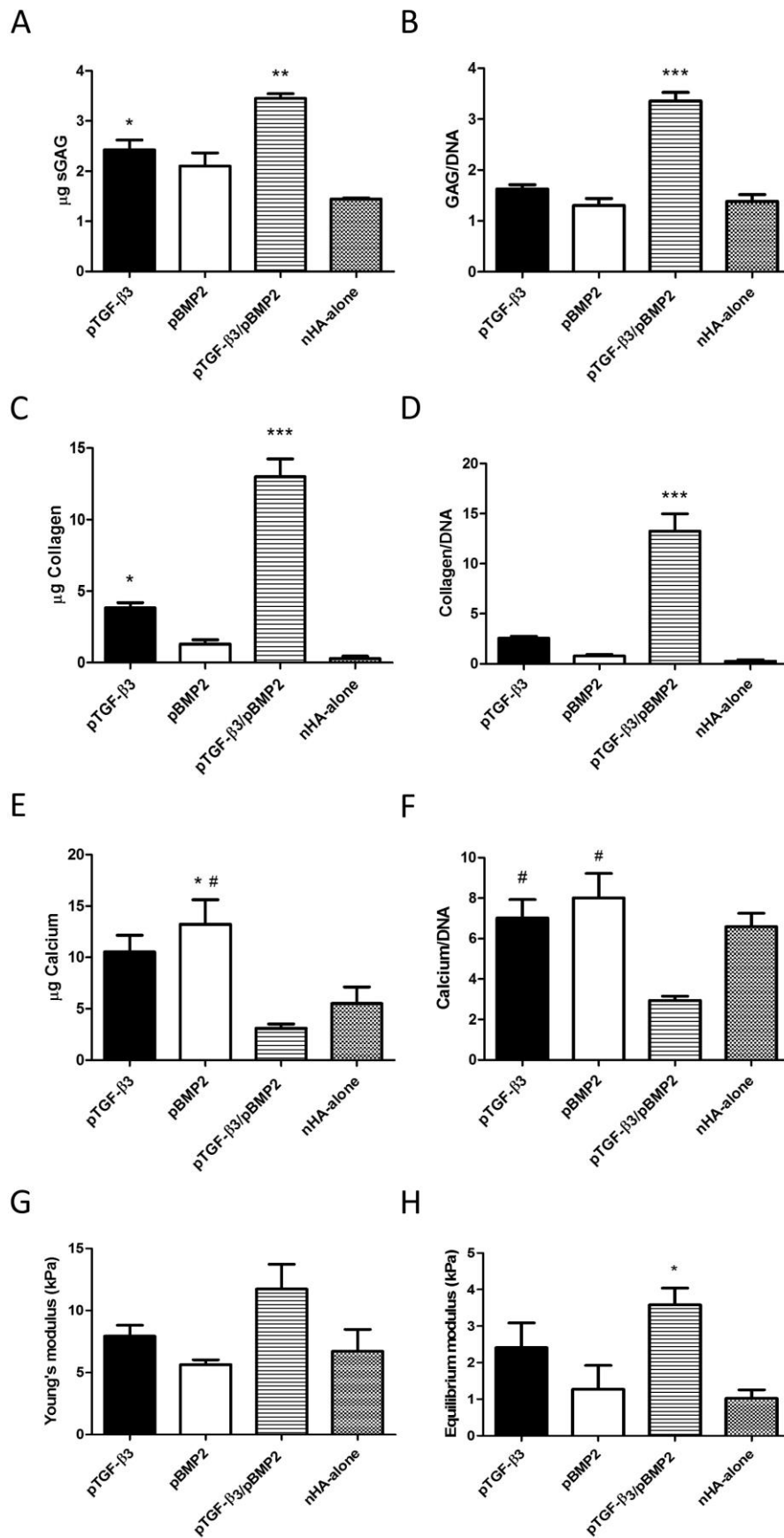


Fig.5.

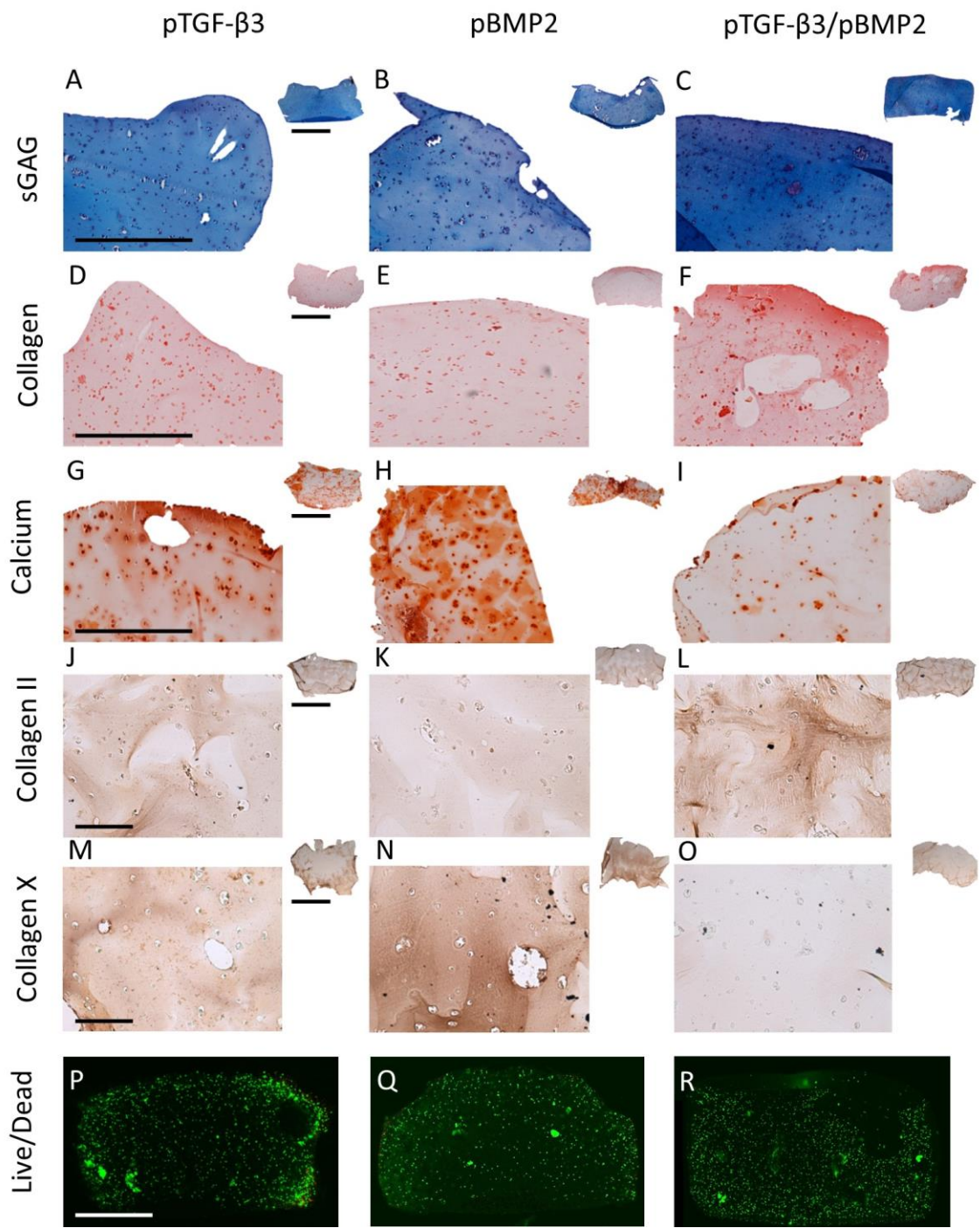
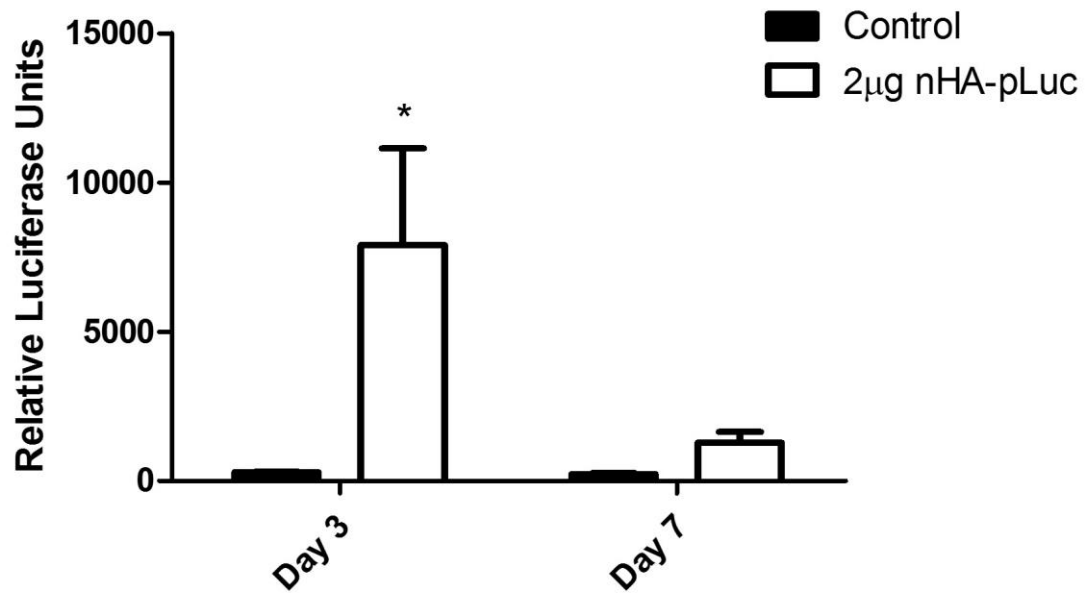


Fig.6.



Supp.Fig.1.