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A chondromimetic microsphere for *in situ* spatially controlled chondrogenic differentiation of human mesenchymal stem cells

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ABSTRACT

Human mesenchymal stem cells (hMSCs) have been identified as a viable cell source for cartilage tissue engineering. However, to undergo chondrogenic differentiation hMSCs require growth factors, in particular members of the transforming growth factor beta (TGF-β) family. While *in vitro* differentiation is feasible through continuous supplementation of TGF-β3, mechanisms to control and drive hMSCs down the chondrogenic lineage in their native microenvironment remain a significant challenge. The release of TGF-β3 from an injectable microsphere composed of the cartilage-associated extracellular matrix molecule hyaluronan represents a readily translatable approach for *in situ* differentiation of hMSCs for cartilage repair. In this study, chondromimetic hyaluronan microspheres were used as a growth factor delivery source for hMSC chondrogenesis. Cellular compatibility of the microspheres (1.2 and 14.1 μm) with hMSCs was shown and release of TGF-β3 from the most promising 14.1 μm microspheres to control differentiation of hMSCs was evaluated. Enhanced accumulation of cartilage-associated glycosaminoglycans by hMSCs incubated with TGF-β3-loaded microspheres was seen and positive staining for collagen type II and proteoglycan confirmed successful *in vitro* chondrogenesis. Gene expression analysis showed significantly increased expression of the chondrocyte-associated genes, collagen type II and aggrecan. This delivery platform resulted in significantly less collagen type X expression, suggesting the generation of a more stable cartilage phenotype. When evaluated in an *ex vivo* osteoarthritic cartilage model, implanted hMSCs with TGF-β3-loaded HA microspheres were detected within cartilage fibrillations and increased proteoglycan staining was seen in the tissue. In summary, data presented here demonstrate that TGF-β3-bound hyaluronan microspheres provide a suitable delivery system for induction of hMSC chondrogenesis and their use may represent a clinically feasible tissue engineering approach for the treatment of articular cartilage defects.

1. Introduction

Controlling mesenchymal stem cell (MSC) differentiation within a complex *in vivo* milieu represents a challenging task requiring finely tuned growth factor delivery to initiate cellular signalling networks [1,2]. Biomaterials have been used as model delivery systems to enhance MSC differentiation. In addition to releasing signalling molecules, biomaterials can provide a substrate for cell attachment. Encapsulation of bioactive molecules can be achieved through chemical crosslinking or simple protein adsorption. This typically exploits direct charge–charge interactions between growth factors and matrices or *via* intermediate proteins such as heparin [3]. Such material approaches include microspheres, hydrogels, scaffolds or combination of scaffolds/hydrogels and microspheres [4,7]. Microspheres derived from materials such as alginate [8] chitosan [9,10] and chondroitin sulphate [11] have been described as appropriate carriers for coordinated release of growth and differentiation factors. By combining TGF-loaded alginate microspheres within a hyaluronic acid hydrogel, Bian et al. demonstrated chondrogenic differentiation of human MSCs (hMSCs) [8]. Similarly, porcine chondrocytes embedded in a chitosan scaffold containing TGF-β1-loaded chitosan microspheres showed increased proliferation and differentiation with increased production of extracellular matrix (ECM) *in vitro* [10]. Chitosan microspheres have also been used to deliver peptides with potential to direct rabbit MSC osteogenic differentiation [9]. Moreover, synthetic microspheres composed of poly(lactic-co-glycolic acid) (PLGA) have shown promise for controlled chondrogenic induction of hMSCs [12,13].

Encapsulation of both cells and growth factors is another method of investigation [14]. This study used hMSCs and an antibody specific for

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bone morphogenetic protein encapsulated in alginate microspheres for bone regeneration. Recently, chemical modification of the surface of PLGA microspheres was shown to affect hMSC differentiation. The addition of amine groups induced hMSC osteogenesis while hydroxyl modification promoted chondrogenic differentiation [15]. The use of microspheres to direct differentiation is not limited to hMSCs [16,17]. For example, gelatin microspheres incorporating different growth factors have been shown to influence lineage commitment of embryonic stem cells [18].

A key advantage of microspheres is the potential for minimally invasive local delivery allowing for optimal construct-tissue integration [19]. Bioactive microspheres, loaded with therapeutic drugs or signalling agents, offer the capacity for controlled and sustained delivery to stimulate tissue regeneration/differentiation [20,21]. As discussed above, one application where the use of microspheres for controlled growth factor delivery may be beneficial is the treatment of articular cartilage defects. hMSCs have been described as an attractive cell source for cartilage regeneration due to their chondrogenic potential and the accessibility of source tissues in comparison to chondrocytes [22]. However, hMSC chondrogenesis *in vitro* requires the repeated addition of TGF-β, usually the 1 or 3 isoform [23]. Whether MSCs are implanted immediately after incorporation into scaffold/hydrogels or pre-differentiated as a bioengineered cartilage construct prior to *in vivo* implantation [24], the availability and/or cost of TGF-β limits the *in vivo* potential of this approach. This limitation is of particular relevance in the context of osteoarthritis where current cartilage tissue engineering applications cannot meet the requirement for repair of large defects or resurfacing of the whole joint [24]. Previous studies using microspheres to promote chondrogenic differentiation of hMSCs have generally focused on materials that are not considered native to articular cartilage [8,9,12]. Herein, we describe the development and characterisation of a chondromimetic hyaluronan (HA) microsphere, a system which we believe is more compatible with cartilage ECM. HA is a critical component of the cartilage ECM and plays a role in lubricating the joint and mechanical support. It also acts to modulate the function of various cells including MSCs and chondrocytes [25,27]. Given that HA is an essential component of matrix, we believe that these microspheres will mimic native cartilage tissue more closely than previously utilised materials. Furthermore, the approach we have taken supports the control of microsphere size [28] compared with previously reported studies [12,29], an important consideration for a clinically desirable intra-articular injectable delivery system.

The release of TGF-β3 from microspheres signifies a translatable approach for *in situ* differentiation of hMSCs. Moreover, the use of microspheres would allow for potential functionalization of their surface with antibodies/peptides for site-specific targeting to enable a more localized concentration of growth factor with reduction in potential off-target effects [30,31]. For the purpose of this study we examined the application of HA microspheres loaded with TGF-β3 to induce chondrogenic differentiation of hMSCs in 3D pellet culture [23] *in vitro* and in an *ex vivo* osteoarthritic cartilage explant system.

2. Materials and methods

2.1. Materials

All reagents were purchased from Sigma–Aldrich unless otherwise stated.

2.2. Preparation and characterisation of hollow HA microspheres

HA microspheres were prepared using a previously reported methodology with modifications [28,32] (Fig. 1A). Briefly, polystyrene beads (PS; 1 ± 0.4 μm and 10.5 ± 2.5 μm GENTAUR, Brussels) were incubated in 2 mg/ml poly(allylamine) in 0.9% sodium chloride (NaCl) for 1 h at room temperature (RT) with agitation followed by incubation with 2 mg/ml HA solution (851 kDa-1190 kDa, LifeCore, USA). Residual nonadsorbed polyelectrolyte was removed by repeated washing with 0.9% NaCl. Following layer deposition, microspheres were stabilised by chemical cross-linking. HA carboxylic groups were activated using water-soluble carbodiimide, 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride and *N*-hydroxysulfosuccinimide, both at concentrations of 0.2 M in 0.1 M 2-(N-morpholino)ethanesulfonic acid, 0.9% NaCl buffer (pH 4.7). Polystyrene cores were removed by treating with tetrahydrofuran. For *in vitro* tracking, microspheres were labelled with fluorescein isothiocyanate–dextran (FITC) (1.0 mg/mL). Collagen (COLL) microspheres, prepared as previously reported were included as a microsphere control in initial experiments [28].

The size and surface uniformity of HA, control PS and COLL microspheres were evaluated by scanning electron microscopy (SEM) using the Hitachi S2600N Variable Pressure SEM with an electron acceleration voltage of 10 kV. 10 μl sample was placed on aluminium stubs, allowed to air dry and gold-coated prior to analysis using a sputter coater. The size distribution of microspheres and surface charge (zeta potential) were measured using the Zetasizer Nano ZS (Malvern instruments, UK).

2.3. Isolation and characterisation of hMSCs

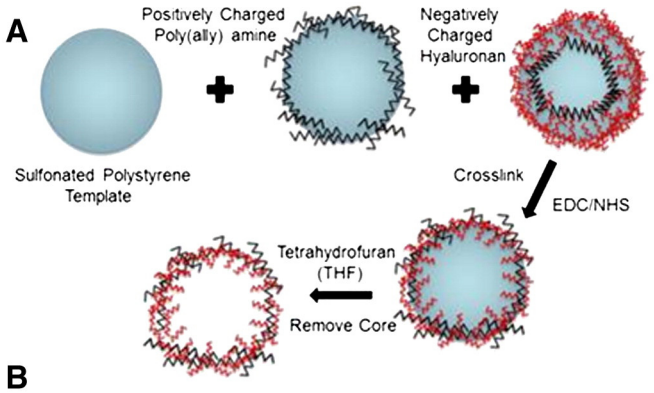
hMSCs were isolated from bone marrow harvested from the iliac crest of healthy donors (18–30 years) with approval from the National University of Ireland Galway and University College Hospital ethics committees and after informed consent. Characterisation of surface receptors was performed using CD105, CD73, CD90 (positive) and CD34, CD45 (negative). Tri-lineage differentiation capacity was determined using standard chondrogenic, adipogenic and osteogenic differentiation assays [33]. MSCs derived from three separate donors, were utilised for experiments and maintained in Minimum Essential Medium Alpha Medium supplemented with 10% foetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S). All cells were cultured at 37 °C and 5% CO_2, unless stated otherwise.

2.4. Interaction of hollow HA microspheres with hMSCs

hMSCs were seeded at 2.5×10^4 cells/cm^2 and incubated with varying concentrations (20, 50, 100, 200 μg/ml) of HA microspheres for 48 h. Cell proliferation and metabolic activity were determined using the Quant-iT™ PicoGreen® dsDNA and alamarBlue® assays (Invitrogen) following manufacturer's instructions. As a control for donor variability, hMSCs grown on tissue culture plastic were included as were PS as an additional control. Metabolic activity was measured by absorbance (550/595 nm) while DNA, isolated by digestion of cells for four hours at 60 °C using papain (1 mg/ml in 50 mM sodium phosphate, pH 6.5, containing 2 mM *N*-acetyl cysteine and 2 mM EDTA) was detected by measuring fluorescence (485/535 nm) on a microplate plate reader (Wallac 1420 Victor 3, Perkin-Elmer Inc.).

2.5. Monocyte activation in response to HA microspheres

Human monocytic leukaemia THP-1 cells were plated at a density of 1×10^5 cells/cm^2 and cultured in RPMI 1640 supplemented with 10% FBS, 5 mM L-glutamine and 1% P/S. Cells were incubated with 200 μg/ml HA, COLL or PS microspheres for 12 h at 37 °C. Stimulation of THP-1 cells with 0.1 μg/ml lipopolysaccharide (LPS) was used as a positive control for monocyte responses. THP-1 cells stimulated with LPS were additionally incubated with HA microspheres to evaluate potential anti-inflammatory properties. Levels of tumour necrosis factor alpha (TNF-α), secreted into the medium, was measured by an enzyme-linked immunosorbent assay (ELISA) (Human TNF-alpha R&D Systems) as per the manufacturer's instructions and absorbance read at 450 nm.



B

Material	Template Size (μm)	Microsphere Size (μm)	Zeta Potential (ZP) (mV)
Hyaluronan	1±0.4	1.2±0.3	-23.4± 1.5
	10.5±2.5	14.1±1.5	-45.6± 4.5
Collagen	1±0.4	1.8±0.2	-16.7±2.5
	10.5±2.5	11±4.3	-20.8±3

C

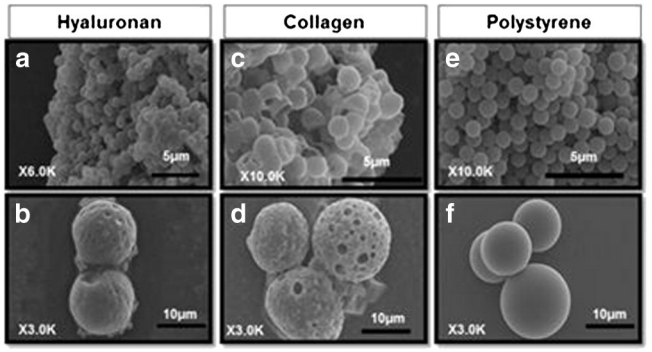


Fig. 1. Fabrication and characterisation of microspheres. (A) Illustration of the fabrication of hollow HA microspheres. (B) Average size distribution and zeta potential of HA and COLL microspheres. (C) Representative SEM images of surface morphology and size distribution of HA microspheres (a, b), COLL microspheres (c, d) and the PS template (e, f). Scale bars 5 μm (a, c, e,) and 10 μm (b, d, f,). Data is representative of the mean ± SD of 3 independent microsphere preparations.

2.6. Cellular interactions of HA microspheres

2.6.1. Flow cytometry

hMSCs were plated at 2.5×10^4 cells/cm^2 and incubated with 20 μg/ml FITC-labelled 1.2 and 14.1 μm HA microspheres for 12, 24 and 48 h at 37 °C. Trypsinised cells were washed twice with PBS to remove extracellular microspheres and resuspended in PBS with 1% bovine serum albumin (BSA). Cell-associated fluorescence was analysed by flow cytometry using a BD FACSCanto™ Flow Cytometer and data processed using FlowJo software. 1×10^4 cells were measured in each sample. The percentage of cells with internalised microspheres was calculated by gating on the number of fluorescence events in the FITC forward scatter signal. Dead cell exclusion was achieved using SYTOX® Dead Cell Stain (Invitrogen).

2.6.2. Confocal microscopy and transmission electron microscopy

To determine cellular internalisation of HA microspheres, 2.5×10^4 cells/cm^2 were seeded on sterile glass cover slips and incubated with 20 μg/ml FITC-labelled microsphere dispersions at 37 °C, 5% CO_2. After 24 h, culture medium was removed and cells washed three times with PBS prior to fixation with 4% formaldehyde for 1 h at RT. Following two PBS washes, cells were incubated with rhodamine phalloidin (Invitrogen) for cytoskeleton staining and 4′6-Diamidino-2-phenylindole (DAPI) slow fade mountant (Invitrogen) for nuclear staining. Stained cells were imaged using the Andor Revolution confocal microscope. For transmission electron microscopy (TEM), 2.5×10^4 hMSCs/cm^2 were incubated with 20 μg/ml of HA and COLL microspheres for 24 h. Trypsinised cells were washed twice with PBS and fixed with 3% glutaraldehyde in freshly prepared 0.2 M sodium cacodylate/HCL buffer (pH 7.2) buffer for 1 h. After washing in the sodium cacodylate buffer the samples were resuspended in 1% osmium tetraoxide for 2 h, washed and samples dehydratated by immersion in graded ethanols and left to dry on the sample holder. Embedding in an epoxy-based resin (Agar Low Viscosity Resin kit) was by sequential exposure to a 50:50 resin/propylene oxide mixture for 4 h, a 75:25 mixture overnight and finally in pure resin for 6 h. After thermo-crosslinking at 65 °C for 48 h samples were observed using the Hitachi H7500 transmission electron microscope (Hitachi Japan).

2.7. In vitro chondrogenic differentiation of hMSCs by TGF-β-bound HA microspheres

TGF-β3 was incorporated onto HA microspheres *via* diffusional loading by directly mixing the hollow HA microspheres with growth factor (400 ng/1 mg microspheres) in PBS containing 1% BSA-0.05% Tween 20. The solution was incubated on a shaker overnight at 4 °C. Microspheres were spun down and supernatant collected for calculation of loading efficiency by ELISA (Human TGF-beta3 ELISA, R&D systems). TGF-β3 release profile of from 14.1 μm HA microspheres was characterised in incomplete chondrogenic medium (ICM) (1 mM Sodium pyruvate, 1% Insulin–Transferrin–Selenium supplement, 4 mg/ml L-Proline, 0.1 mM Ascorbic acid 2-Phosohate, dexamethasone, Dulbecco's Modified Eagle Medium, high glucose (DMEM-HG), 1 mM Antibiotic/Antimycotic 100× solution) at 37 °C. 2.5 mg loaded microspheres were incubated with 200 μl ICM and the supernatant was collected and replaced at various time points. Cell viability of chondrogenic pellets after 21 days was analysed using calcein-acetoxymethylester (Calcein AM)/ethidium Homodimer-1 (EthD-1) fluorescence staining. Pellets were washed twice with PBS and then incubated with Calcein-AM (1 μM) and EthD-1 (2 μM) for 30 min at RT. After washing, pellets were analysed by fluorescence microscopy (Zeiss LSM 510 Axiovert inverted confocal microscope).

For chondrogenesis, 2.5×10^5 hMSCs were cultured in either ICM or complete chondrogenic media (CCM) (ICM supplemented with 10 ng/ml TGF-β3) or with TGF-β3-loaded (250 ng) or unloaded HA microspheres. Cells were maintained in hypoxia (2% O_2) for 14 or 21 days. Medium was changed every two days. For histological and immunohistochemical evaluation, chondrogenic pellets were sequentially dehydrated in graded ethanol solutions (70–100%), paraffin-embedded, cut into 5 μm-thick sections and mounted on slides. Sections were stained with 0.03% Toluidine blue for 5 min at 60 °C to visualise sulfated proteoglycans. Immunohistochemistry was performed as described previously [34] using (Abcam; 1/100ab2601) for detection of type II collagen. Negative controls were without primary antibody. Glycosaminoglycan (GAG) and DNA were measured after digestion in a papain solution (0.0025 mg/ml) at 60 °C overnight. Digests were assayed for DNA using the Picogreen® assay and GAG content was determined using a dimethylmethylene blue dye assay. The total amount of GAG was normalised against the total amount of DNA.

2.8. Quantitative real-time polymerase chain reaction (qRT-PCR)

Gene expression was analysed by quantitative qRT-PCR. Total RNA was extracted using Tri-Reagent (Invitrogen) after grinding with an Eppendorf® micropestle and quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, DE). Each PCR included 10 μl 2X SensiFAST SYBR Hi-ROX One-Step Mix, 0.4 μl RiboSafe RNase

Inhibitor (0.2 U/μl), 0.5 μl of forward and reverse primers (400nM), 3.6 μl of DEPC-H20 and 5 μl of template (20 ng RNA). Real-time PCR reactions were performed using SYBR Green chemistry on a StepOnePlus ™ Real-Time PCR System (Applied Biosystems, CA); conditions were 45 °C for 10 min, 95 °C for 10 min and 45 cycles of 95 °C, 60 °C and 72 °C at 15 s each. Collagen X and aggrecan primers were purchased (Qiagen, CA). Collagen II (5′-3′TCCCTTTGGTCCTGGTTGCC and ATCTGC CCAACTGACCTCGCCA) and drosha (5′-3′TCCATGCACCAGATTCTCCTG and TACGGACAGAGCTTGGTTTCG) primers were designed and purchased from Sigma. Cycle threshold (*CT*) values were established and normalised to the endogenous control drosha, as described previously for MSC differentiation [35]. Results were expressed as fold change compared to ICM MSC control.

2.9. Ex vivo osteoarthritic cartilage explant culture

Fresh human articular cartilage samples were obtained under institutionally approved protocols from patients with end-stage osteoarthritis who were undergoing total knee arthroplasty at Merlin Park Hospital, Galway. Full thickness cartilage explants (1–2 mm thick and 3 mm diameter) were taken by biopsy punch (Pan Vet) from the medial tibial plateau. Cartilage explants were allowed to equilibrate at 37 °C for 48 h in DMEM with 10% FBS and subsequently for 24 h in ICM in a 2% solidified agarose well. ICM was removed from the explants and hMSCs alone, hMSCs with HA microspheres or hMSCs with TGF-β3-loaded HA microspheres were added. Groups were either cultured in ICM or CCM in hypoxia for 21 d. Cartilage explants were prepared for histological analysis and stained with Toluidine blue to assess production of sulfated proteoglycan as described above.

2.10. Statistical analysis

GraphPad Prism® software Inc. (version 5.03) was used for statistical analysis. Significance was assessed using one-way or two-way ANOVA followed by Tukey or Bonferroni post hoc analysis, respectively. Error bars represent the mean ± standard deviation or standard error of the mean when indicated. *p*-values ≤0.05 were considered statistically significant.

3. Results

3.1. Characterisation of HA microspheres

HA microspheres were successfully fabricated (Fig. 1A) and SEM was utilised to assess the structure compared to control COLL microspheres and the PS template. Microspheres exhibited a uniform, spherical shape with HA microspheres using the 10.5 ± 2.5 μm template having a mean diameter of 14.1 ± 1.5 μm and 1.2 ± 0.3 μm using the 1 ± 0.4 μm template. The microspheres had a relatively smooth surface with evidence of some porous indentations due to the layer deposition compared to the smooth polystyrene beads. Both 1.2 μm and 14.1 μm HA microspheres showed negative zeta potential values of −23.4 ± 1.5 and −45.6 ± 4.5 respectively, indicating that the larger HA microspheres may be more stable (Fig. 1B and C).

3.2. Cellular response to HA microspheres

In vitro compatibility studies were performed to determine the possible toxicity of HA microspheres when exposed to hMSCs. Cell viability in the presence of HA microspheres at varying concentrations was assessed relative to cells exposed to PS and cells alone as controls. HA microspheres (1.2 or 14.1 μm) had no effect on hMSC proliferation. Although DNA levels were higher in cultures treated with 100 and 200 μg/ml 14.1 μm microspheres, respectively, this was not statistically significant (Fig. 2A and B). Metabolic activity of cells grown in the presence of the HA microspheres compared to cells alone was not statistically decreased with a reduction of less than 20% across all concentrations tested (Fig. 2C and D). Furthermore, to assess whether HA microspheres might elicit a pro-inflammatory response, the release of TNF-α from THP-1 cells was analysed in the supernatant following incubation with microspheres for 12 h. Similarly, no significant increases in TNF-α were detected. Since high molecular weight HA (>800 kDa) has been associated with anti-inflammatory and chondroprotective effects [36,37], we assessed if HA microspheres could reduce an inflammatory response using LPS stimulated THP-1 cells. Although the addition of 14.1 μm HA microspheres appeared to reduce TNF-α production by LPS stimulation by 21%, results were not statistically significant (Fig. 2E). These results confirm the low toxicity of the microspheres to hMSCs. Furthermore, they do not elicit an inflammatory response, as demonstrated by the inability of the microspheres to activate THP-1 monocytes.

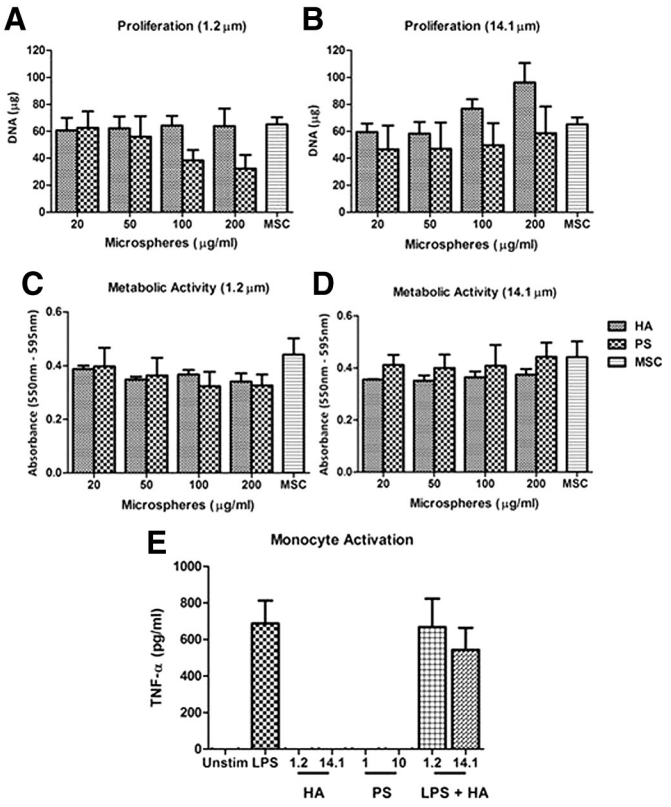


Fig. 2. Cellular compatibility of HA microspheres. Influence of HA microsphere size and concentration on proliferative state (A, B) and metabolic activity (C, D) of hMSCs. (E) Production of TNF-α from unstimulated and LPS activated (0.1 μg/ml) THP-1 cells. Data represents the mean ± SEM of 3 hMSC donors (A–D) and mean ± SD of 3 independent experiments (E). Two-way ANOVA (A–D) and one-way ANOVA (E), $p > 0.05$.

3.3. Cellular uptake/in vitro cellular association of HA microspheres

Confocal microscopy, flow cytometry and TEM were used to assess uptake of HA microspheres by hMSCs. COLL microspheres (1.8 and 11 μm) were included as controls. Microspheres were fluorescently labelled with FITC to facilitate *in vitro* tracking. Confocal images illustrated the interaction between hMSCs and microspheres (Fig. 3Ac, d, g and h) while TEM confirmed the presence of microspheres intracellularly (Fig. 3Aa, b, e and f). The smaller HA and COLL microspheres were detected within the cells, while larger microspheres were not taken up to the same extent, although some were found intracellularly (red arrows). Quantification of cellular uptake was carried out using flow cytometry. In line with TEM analysis, there was a significant increase in uptake of smaller microspheres across all time points with at least 85% more uptake of 1.8 μm COLL compared to 1.2 μm HA microspheres.

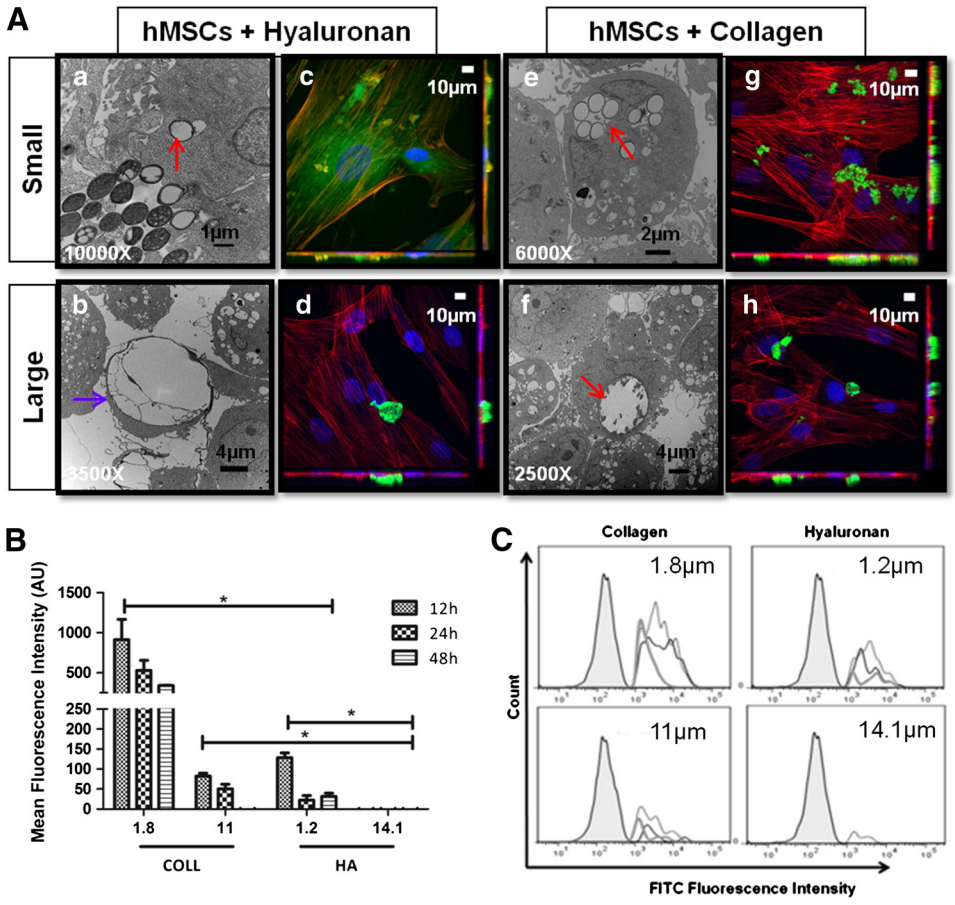


Fig. 3. Determination of cellular uptake of HA and COLL microspheres by hMSCs. (A) Confocal microscopy images (c, g, d, h) and TEM analysis (a, e, b, f) illustrate the interaction and uptake of HA and COLL microspheres by hMSCs at 12 h. Blue, Nucleus; Red, Cytoskeleton; Green, Microspheres. Microspheres located intracellularly (red arrows) and extracellularly (purple arrow). (B) Flow cytometry analysis of hMSCs incubated with HA and COLL microspheres (1.8 and 11 µm) for 12, 24 and 48 h. Mean fluorescence intensity was estimated for each group and normalised to hMSCs alone. (C) Representative fluorescence intensity histograms for HA and COLL microspheres. Data is representative of the mean ± SD of 3 hMSC donors. Two-way ANOVA performed, $^{*}p < 0.05$ COLL 1.8 *vs.* COLL 11 µm, COLL 11 *vs.* HA 14.1 µm and HA 1.2 *vs.* HA 14.1 µm (across all times).

Similarly, there was a significant increase in uptake of 11 µm COLL compared to 14.1 µm HA and also 1.2 µm HA compared with 14.1 µm HA microspheres (Fig. 3B, C). These results suggested that the 14.1 µm HA microspheres were more appropriate for growth factor delivery and extracellular targeting and thus were selected and used in further *in vitro and ex-vivo* growth factor loading studies.

3.4. HA microspheres as delivery vehicles for hMSC chondrogenesis

The ability of TGF-β3-loaded HA microspheres to promote chondrogenic differentiation of hMSCs in pellet culture was assessed (Fig. 4A). A high loading efficiency of TGF-β3, approximately 80%, was observed using 14.1 µm HA microspheres. The TGF-β3 release profile was investigated in a pellet culture system with or without hMSCs. Fig. 4B shows the cumulative release of TGF-β3 into ICM medium. Inclusion of the cells had no effect on the release of the growth factor. An initial burst release extending up to 4 days followed by low but sustained release to day 10 was observed; a release profile similar to Bian et al., 2012. Analysis indicated that this microsphere delivery system followed zero order kinetics. Assessment of TGF-β3 levels released from the microspheres on days 5–10 indicated that 4 to 10 ng/ml growth factor was detected (Fig. 4C). For chondrogenesis to occur *in vitro*, hMSCs are exposed to 10 ng/ml fresh TGF-β3 every 2 days [23]. Using these conditions, 3–8 ng/ml TGF-β3 was detected in the medium throughout the experiment (Supplementary Fig. 1) indicating that the levels released from TGF-β3-loaded microspheres after 4 days were sufficient to promote chondrogenesis.

Viability of hMSC ± TGF-β3-loaded HA microsphere pellets was assessed using live/dead stain after 21 d culture. There was no difference in cell death between the MSC control group and TGF-β-loaded HA microsphere group (Fig. 4D).

3.5. In vitro chondrogenesis of hMSC with TGFβ3-loaded HA microspheres

To evaluate the bioactivity of released TGF-β3, hMSCs were cultured with TGF-β3-loaded HA microspheres and assessed for their ability to promote *in vitro* chondrogenesis after 21d culture. Histological sections were stained for proteoglycan and assessed by blue to pink staining (metachromasia) with toluidine blue (Fig. 5a–f) and brown immunostain indicative of collagen type II (Fig. 5g–l) to determine relative amounts and distribution within the pellets. Groups included hMSC alone, hMSC with HA microspheres and hMSC with TGF-β3-loaded HA microspheres treated with ICM with no TGF-β3 added throughout the culture period, or CCM as a positive control with 10 ng/ml TGF-β3 added every 2 days. In ICM, type II collagen and toluidine blue staining were negative in both control hMSC (Fig. 5Aa, g) and HA microsphere alone groups (Fig. 5Ab, h) as demonstrated by no brown stain for collagen type II and weak blue stain for proteoglycan. The most intense matrix staining was observed with the TGF-β3-loaded microsphere groups (Fig. 5Ac, i), which showed strong metachromasia (blue to pink) with toluidine blue and immunostained brown for collagen type II. This was comparable to the positive control of hMSCs cultured with free TGF-β3 every 2 d (Fig. 5Ad, j). Positive staining was also detected in the additional, positive control groups

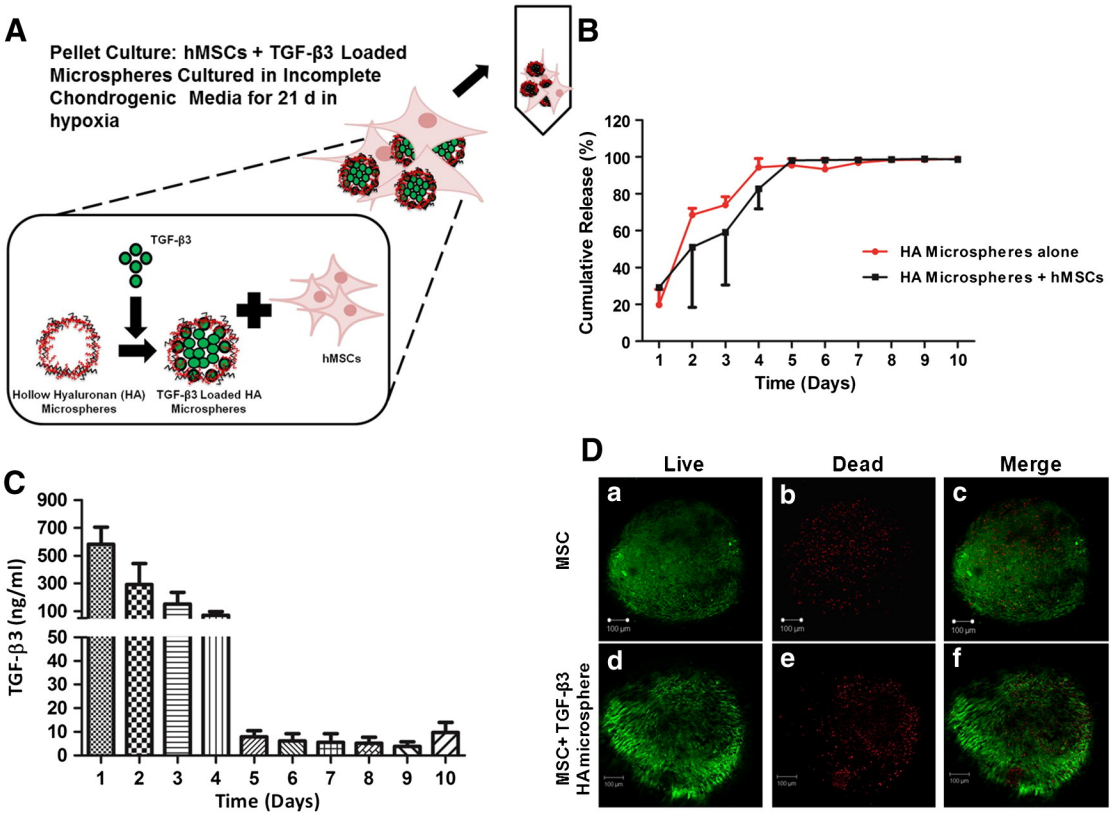


Fig. 4. Loading, viability and release of TGF-β3 from hollow HA microspheres. (A) Illustration of the TGF-β3-loaded HA microsphere pellet culture system. (B) Cumulative release profile of TGF-β3 from microspheres alone and in the presence of hMSCs over 10 d in ICM medium. (C) Concentration of TGF-β3 released from HA microspheres cultured with hMSCs in ICM for 10 days. (D) Viability of chondrogenic pellets cultured for 21 d with (d, e, f) and without (a, b, c) TGF-β3-loaded microspheres in ICM medium. Data is presented as the mean ± SD of 3 technical replicates for experiments performed with 2 hMSC donors and 2 individual microsphere preparations. Scale bar, 100 μm.

containing HA microspheres and TGF-β3-loaded HA microspheres cultured in CCM (Fig. 5Ae, k, f, l).

Quantitative analysis of GAG in each group demonstrated that the amount of deposition as well as DNA content was substantially higher in the hMSC group with TGF-β3-loaded HA microspheres cultured in ICM compared to hMSC and hMSC with HA microspheres alone groups ($P < 0.05$, Fig. 5B, C). In addition, significantly more GAG was detected compared to the hMSC cultured in CCM group (Fig. 5B). Results following normalisation of GAG to the DNA content again support staining results. Total GAG/DNA content in the group that received TGF-β3-loaded-HA microspheres cultured in ICM was significantly higher than that of the hMSC only and hMSC with HA microsphere groups cultured in the same conditions, where negligible amounts of GAG/DNA were detected. There was also significantly more GAG/DNA content in the groups that received HA microspheres and TGF-β3-loaded-HA microspheres compared with hMSCs alone cultured in CCM, where 10 ng/ml TGF-β3 was added every 2 d (Fig. 5D). This suggests a beneficial role of HA microspheres in promoting GAG synthesis. Loading HA microspheres with 250 ng/ml TGF-β3 had no additive effect over HA microspheres alone in CCM. This group exposed to fresh TGF-β3 every 2 days as well as growth factor released from HA microspheres did not show increased GAG deposition compared to the TGF-β3 loaded HA microspheres cultured in ICM. These results suggest that the initial burst release followed by sustained release of TGF-β3 from the HA microspheres was sufficient to initiate and maintain chondrogenesis of hMSCs.

Next we examined whether proteoglycan production was associated with the induction of chondrogenic transcripts. Quantification of markers was performed on RNA extracted from all test groups, cultured in both ICM and CCM for 14 d. Collagen type II was significantly upregulated in the group that received TGF-β3-loaded-HA microspheres cultured in ICM compared to hMSCs alone cultured in the same conditions (385 fold increase) (Fig. 6A). Additionally, there was a significant increase in collagen type II in the hMSC groups cultured in CCM with TGF-β3-loaded-HA microspheres compared to hMSCs alone cultured in CCM. The same trend was observed with aggrecan, with a significant increase detected in the group that received TGF-β3-loaded-HA microspheres cultured in ICM compared to hMSCs alone (Fig. 6B). Significantly, there was less collagen X in this group compared to the hMSC cultured in CCM group (pellets receiving TGF-β3 every 2 d), indicating the formation of pellets with a more hyaline-like articular cartilage phenotype (Fig. 6C). These results demonstrate that culture of hMSCs in the presence of TGF-β3-releasing HA microspheres enabled *in vitro* differentiation to chondrocytes in addition to promoting the secretion of extracellular matrix components, characteristic of hyaline cartilage.

3.6. Ex-vivo culture of human osteoarthritic cartilage explant with HA microspheres

The ability of HA microspheres loaded with TGF-β3 to support hMSC chondrogenic differentiation was evaluated in a human osteoarthritic explant culture system. Cartilage explants were exposed to ICM in a 2% agarose well (Fig. 7A) in the presence of hMSCs alone (a, b), hMSCs with HA microspheres (c, d) or with HA microspheres loaded with TGF-β3 (e, f). Explants with hMSCs and exposed to fresh TGF-β3 every 2 days were included as positive controls (g, h). Sections were stained with toluidine blue for detection of proteoglycans. Exogenously added hMSCs (*) were seen as blue flattened cells and HA microspheres (black arrow) as white circles along the surface of the damaged articular cartilage or within characteristic osteoarthritic fibrillations/clefts. An increase in GAG deposition in exogenously added hMSCs (f, dotted red

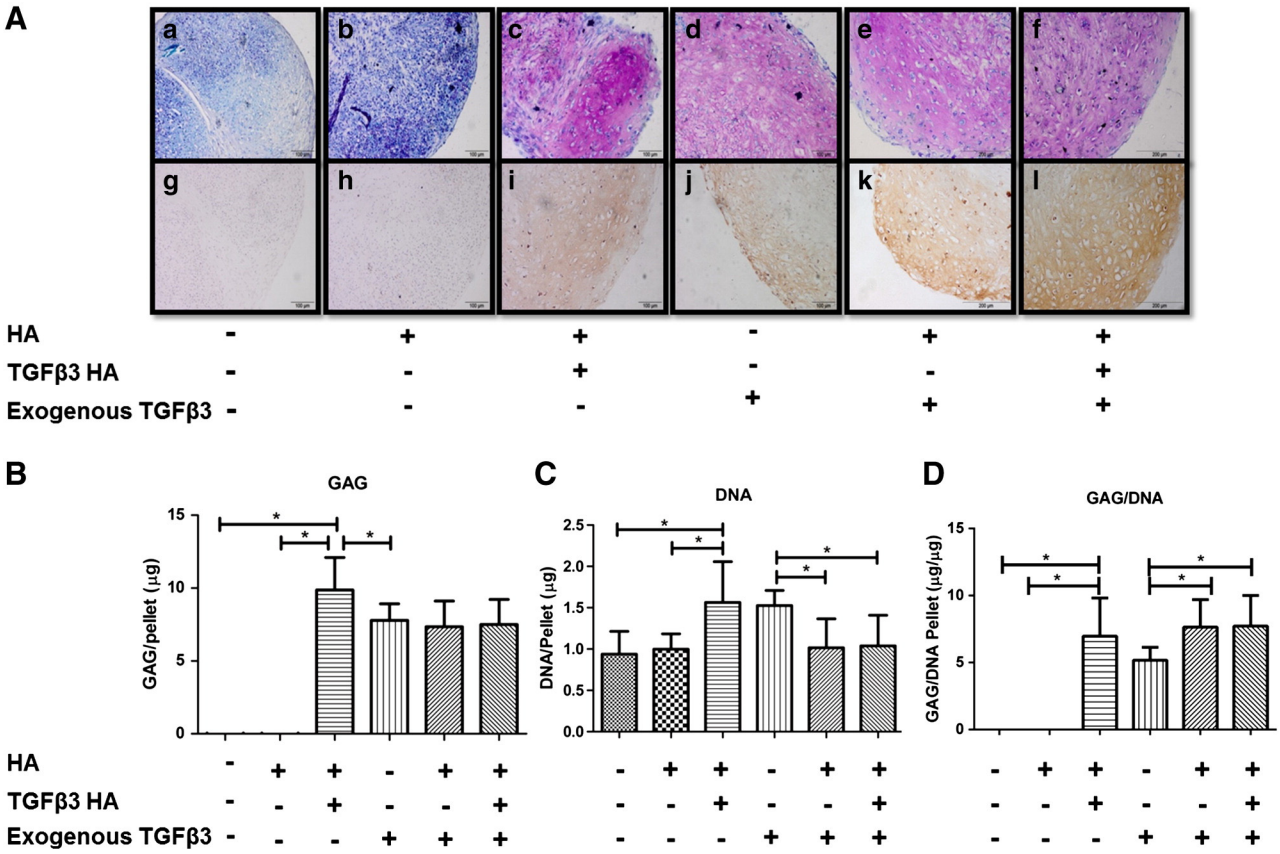


Fig. 5. *In vitro* chondrogenesis of hMSCs by release of TGF-β3 from HA microspheres. (A) Representative images of 21 d chondrogenic pellets sectioned and stained with toluidine blue (a–f) and collagen type II (g–l). Negative control groups included hMSC alone (a, g) and hMSC + HA microspheres (b, h) in ICM medium. Positive staining for GAG and collagen type II was evident in the test group, hMSC + TGF-β3-loaded HA microspheres in ICM (c, i). Positive control groups included hMSC, hMSC + HA microspheres and hMSC + TGF-β3-loaded HA microspheres cultured in CCM throughout (d-l). Proteoglycan content was quantified by measuring (B) GAG (C) DNA and (D) GAG/DNA ratio. Results indicated a significant increase in GAG and GAG/DNA deposition in the test group (hMSC + TGF-β3-loaded HA microspheres in ICM) compared to the negative control group. Scale bars, 200 μm (e, f, k, l) and 100 μm (a–d, g–j). HA = Hyaluronan microspheres, TGF-β3 HA = TGF-β3 loaded HA microspheres; Exogenous TGF-β3 = 10 ng/ml TGF-β3 added freshly to culture every 2 d. Data is representative of the mean ± SD of 3 hMSC donors. One-way ANOVA and post-hoc Tukey post-test performed, $^*p < 0.05$.

arrow) and resident chondrocytes (f, solid red arrows) was seen after exposure to TGF-β3-loaded HA microspheres for 21 d. This was comparable to the positive controls where explants were exposed to freshly-added TGF-β3 throughout the culture period (g, h).

4. Discussion

There is a growing demand for the development of injectable systems for the regeneration of articular cartilage defects. The use of microspheres composed of HA could form the basis for a chondroconductive environment for hMSCs with release of TGF-β3 from this reservoir acting to initiate chondrogenic differentiation of hMSCs. This delivery system could also potentially act as a recruitment signal for endogenous chondroprogenitor cells. The present study describes the preparation of hollow HA microspheres through electrostatic layer-by-layer self-assembly. We validated the microspheres as a chondromimetic platform for delivery of growth factors to promote chondrogenesis of hMSCs *in vitro* and *ex vivo*. The basic principle behind the methodology used to fabricate these microspheres has been described previously and is based on the sequential and repeated electrostatic adsorption of positively and negatively charged polymers onto a charged template [38]. In addition to forming reproducible microspheres, another advantage of the template method is the ability to remove the core for the incorporation of bioactive molecules such as growth factors [28,39,40].

Given that microsphere size and shape is a critical parameter affecting cellular interaction and uptake [41], we compared microspheres with regard to cellular uptake. As internalisation was observed with smaller microspheres, the larger 14.1 μm HA microspheres were selected to investigate their capability as a growth factor carrier for TGF-β3. Furthermore, HA 14.1 μm microspheres were more stable. The selection of this size range is similar to the study by Ko et al., who utilised PLGA microspheres with a mean size of 14.5 ± 0.81 μm for the intra-articular delivery of an anti-inflammatory agent for modulation of osteoarthritis. In this study, necropsy of liver, spleen, kidney and bone marrow was carried out with no gross or histological differences noted following injection of these size particles [42]. This would suggest that this size range does not cause systemic toxicity. We have shown that HA microspheres are compatible with hMSCs and do not activate monocytes, an indicator of inflammation. This data agrees with other studies where HA was employed as a delivery scaffold with no adverse effects noted [43,44]. HA has also been used as an intra-articular treatment for management of pain in osteoarthritis patients for a number of years with a recent systematic review indicating positive effects [45].

TGF-β3 has been identified as an effective stimulator of cartilage tissue development and regeneration, and functions in regulation of several cellular activities including proteoglycan metabolism [46,47]. For this reason, it was selected as a candidate for controlled induction of chondrogenic differentiation of hMSCs. Additionally, members of the TGF-β family have been shown to enable the recruitment of hMSCs and progenitors *in vivo* [48,49]. Zeta potential results indicated that the HA microspheres were negatively charged suggesting that they

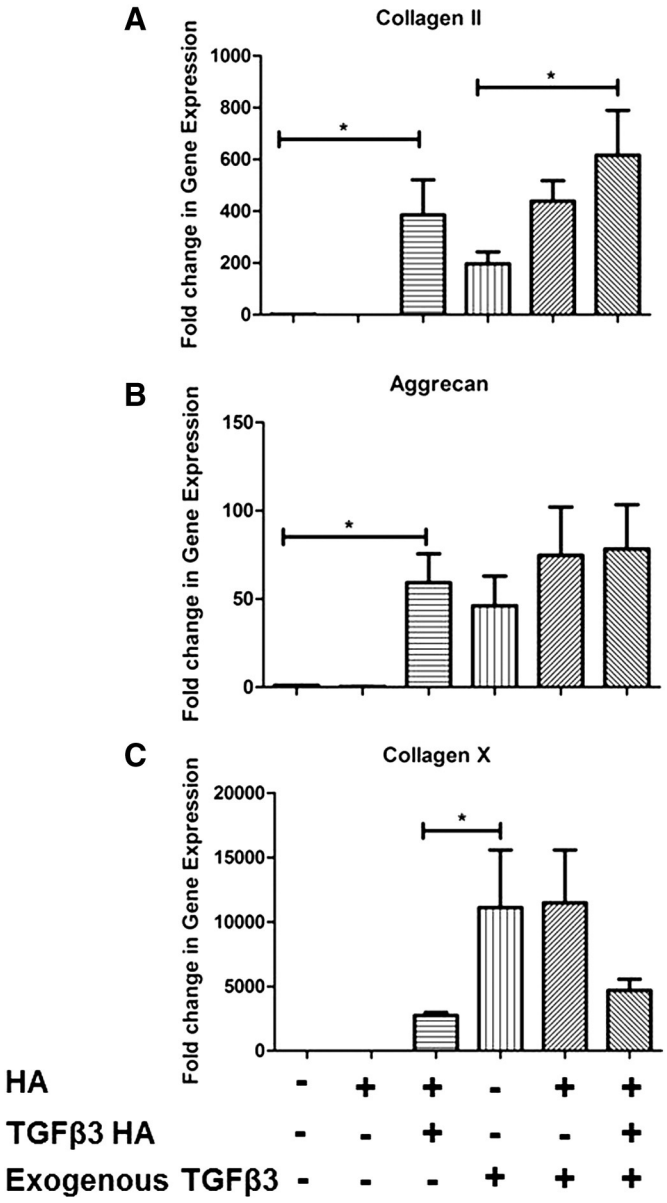


Fig. 6. Effect of TGF-β3-loaded HA microspheres on gene expression of chondrogenic markers. Levels of gene expression for chondrogenic markers (A) collagen type II, (B) aggrecan and (C) collagen type X were investigated by real-time PCR after 14 d in culture. Expression was normalised to drosha and expressed as fold change compared to hMSCs cultured in ICM. HA = Hyaluronan Microspheres, TGF-β3 HA = TGF-β3 loaded HA microspheres; Exogenous TGF-β3 = 10 ng/ml TGF-β3 added freshly to culture every 2 d. Data is representative of the mean ± SEM for 3 hMSC donors, (*$p < 0.05$).

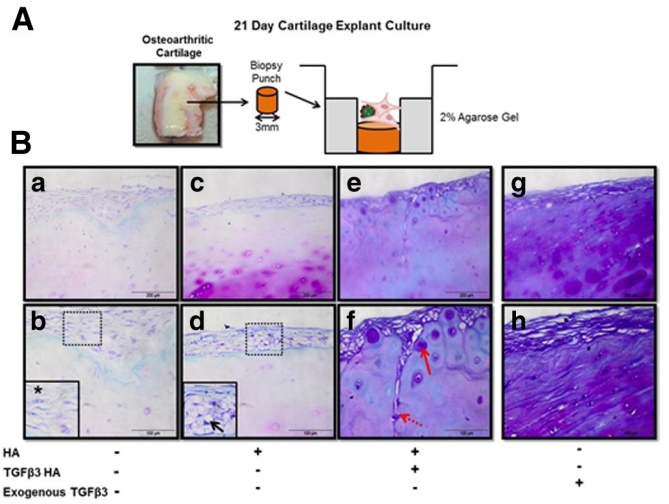


Fig. 7. *Ex vivo* culture of osteoarthritic human cartilage explants. (A) Schematic representing the explant culture format with cartilage biopsies embedded in 2% agarose gel. (B) Explants were exposed to hMSCs alone (a, b), hMSCs with HA microspheres (c, d), and hMSCs with TGF-β3-loaded HA microspheres (e, f) in ICM. The positive control explants were treated with hMSC alone and cultured in CCM with fresh 10 ng/ml TGF-β3 every 2 d (g, h). HA = Hyaluronan Microspheres, TGF-β3 HA = TGF-β3 loaded HA microspheres, Exogenous TGF-β3 = 10 ng/ml TGF-β3 added freshly to culture every 2 d. Representative images of n = 3 explants with lower panels showing areas of the explant surface in a, c, e and g. Scale bars, 200 μm (a, c, e, g) and 100 μm (b, d, f, h). Insets (b, d) represent magnified images of the areas highlighted by the dotted rectangles to highlight cell (b) and microsphere morphology (d). *, hMSCs; black arrow, HA microspheres; dotted red arrow, MSC-like cells; solid red arrow, resident chondrocytes.

would be optimal for electrostatically binding the cationic TGF-β3. This was verified by the high loading efficiency, similar to that previously described for PLGA and chitosan microspheres [10,50]. Cumulative release data suggested a strong initial burst release from the microspheres followed by a sustained release of TGF-β3. We believe TGF-β3 loaded HA microspheres not only provided a micro-milieu suitable for initiating the signals required for chondrogenesis of hMSCs, but enabled release of sufficient TGF-β3 to maintain differentiation over time. Although, not investigated in this work, incorporation of microspheres within hydrogels can further delay the release profile of growth factors, if desirable [51]. Previous studies have suggested that short term exposure of TGF-β3 may be sufficient to initiate the key regulatory events for chondrogenesis to occur [52,53]. The addition of high levels of TGFβ in the initial days of hMSC chondrogenic differentiation resulted in similar levels of chondrogenesis compared to continuous culture [54]. Similarly, bovine MSCs that were cultured in a methacrylated hyaluronic acid tissue construct with 100 ng/mL of TGF-β3 for 7 days demonstrated superior mechanical and biochemical properties, exceeding groups that received continuous TGF-β3 delivery [55]. This suggests that the commitment of MSCs down the chondrogenic lineage is influenced at the early stage of differentiation and that an initial burst release seen in this study using HA microspheres may be advantageous.

In their native environment, many endogenous growth factors are found bound within the ECM, where they are stored until required; thus ECM plays an important role in regulating growth factor release and activation [56]. Although the mechanism of action of HA microspheres was not directly investigated in this study, we postulate that it is both the presence of HA and TGF-β3 that is successfully supporting chondrogenesis. This is in accordance with previous literature that has shown stem cell interactions with HA promote expression of early chondrogenic markers, such as the cartilage transcription factor SOX9 and collagen type II. Inclusion of HA also enhanced TGF-β-induced chondrogenic differentiation of hMSCs in an alginate layer system [57,60]. This might explain why incorporation of HA microspheres in normal chondrogenic conditions had a positive synergistic effect on differentiation, as seen from the increased GAG/DNA ratio. It is possible that exogenously added TGF-β3 bound to HA microspheres in the developing pellet and allowed for more efficient availability of growth factor. Moreover, this retention may be particularly important for growth factors with a short half-life such as TGF-β3 [61] and would be an advantage for *in vivo* applications by providing protection from circulating proteases and enzymes.

Unregulated TGF-β3 signalling can be detrimental to some tissues and plays a known role in many pathologies including liver fibrosis [62]. In chondrogenesis, release of high levels of TGF-β3 throughout the process is not necessarily desirable and may drive cells to become hypertrophic or induce fibrosis as previously demonstrated in a murine knee joint [63,65]. We have shown that compared to normal chondrogenic culture conditions the use of TGF-β3-loaded HA microspheres results in significantly less collagen type X expression, a marker of undesirable late-stage chondrocyte hypertrophy and terminal

differentiation. This result is in accordance with similar studies [8] but also surpasses previously reported results [50,66]. Thus, the intra-articular injection and release of TGF-β3 from delivered microspheres could prove advantageous in avoiding potential side effects described previously [67]. Herein, we present *in vitro* and *ex-vivo* evidence to support the use of TGF-β3-loaded HA microspheres for *in situ* differentiation of hMSCs. The results indicate that in addition to being compatible with hMSCs, these microspheres can provide a matrix for *in vitro* chondrogenesis and may have potential to differentiate endogenously-recruited mesenchymal progenitors. Ultimately, the use of growth factor-releasing chondromimetic HA microspheres can be envisaged as a one-step clinically translatable protocol for cartilage repair, presenting many advantages over current treatments such as autologous cartilage implantation, an invasive two step procedure. Nevertheless, further studies are warranted to assess this promising delivery platform *in vivo*.

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References

[1] F. Ng, S. Boucher, S. Koh, K.S. Sastry, L. Chase, U. Lakshmipathy, C. Choong, Z. Yang, M.C. Vemuri, M.S. Rao, V. Tanavde, PDGF, TGF-beta, and FGF signaling is important for differentiation and growth of mesenchymal stem cells (MSCs): transcriptional profiling can identify markers and signaling pathways important in differentiation of MSCs into adipogenic, chondrogenic, and osteogenic lineages, Blood 112 (2008) 295–307.
[2] M.F. Pittenger, A.M. Mackay, S.C. Beck, R.K. Jaiswal, R. Douglas, J.D. Mosca, M.A. Moorman, D.W. Simonetti, S. Craig, D.R. Marshak, Multilineage potential of adult human mesenchymal stem cells, Science 284 (1999) 143–147.
[3] J.S. Park, K. Na, D.G. Woo, H.N. Yang, K.H. Park, Determination of dual delivery for stem cell differentiation using dexamethasone and TGF-beta3 in/on polymeric microspheres, Biomaterials 30 (2009) 4796–4805.
[4] T. Deng, S. Huang, S. Zhou, L. He, Y. Jin, Cartilage regeneration using a novel gelatin–chondroitin–hyaluronan hybrid scaffold containing bFGF-impregnated microspheres, J. Microencapsul. 24 (2007) 163–174.
[5] H. Fan, Y. Hu, L. Qin, X. Li, H. Wu, R. Lv, Porous gelatin–chondroitin–hyaluronate tricopolymer scaffold containing microspheres loaded with TGF-beta1 induces differentiation of mesenchymal stem cells *in vivo* for enhancing cartilage repair, J. Biomed. Mater. Res. A 77 (2006) 785–794.
[6] K. Park, J.S. Park, D.G. Woo, H.N. Yang, H.M. Chung, K.H. Park, The use of chondrogenic differentiation drugs to induce stem cell differentiation using double bead microsphere structure, Biomaterials 29 (2008) 2490–2500.
[7] A.J. DeFail, C.R. Chu, N. Izzo, K.G. Marra, Controlled release of bioactive TGF-beta 1 from microspheres embedded within biodegradable hydrogels, Biomaterials 27 (2006) 1579–1585.
[8] L. Bian, D.Y. Zhai, E. Tous, R. Rai, R.L. Mauck, J.A. Burdick, Enhanced MSC chondrogenesis following delivery of TGF-beta3 from alginate microspheres within hyaluronic acid hydrogels *in vitro* and *in vivo*, Biomaterials 32 (2011) 6425–6434.
[9] X. Niu, Q. Feng, M. Wang, X. Guo, Q. Zheng, Porous nano-HA/collagen/PLLA scaffold containing chitosan microspheres for controlled delivery of synthetic peptide derived from BMP-2, J. Control. Release 134 (2009) 111–117.
[10] S.E. Kim, J.H. Park, Y.W. Cho, H. Chung, S.Y. Jeong, E.B. Lee, I.C. Kwon, Porous chitosan scaffold containing microspheres loaded with transforming growth factor-beta1: implications for cartilage tissue engineering, J. Control. Release 91 (2003) 365–374.
[11] J.J. Lim, T.M. Hammoudi, A.M. Bratt-Leal, S.K. Hamilton, K.L. Kepple, N.C. Bloodworth, T.C. McDevitt, J.S. Temenoff, Development of nano- and microscale chondroitin sulfate particles for controlled growth factor delivery, Acta Biomater. 7 (2011) 986–995.
[12] E.K. Moioli, L. Hong, J. Guardado, P.A. Clark, J.J. Mao, Sustained release of TGFbeta3 from PLGA microspheres and its effect on early osteogenic differentiation of human mesenchymal stem cells, Tissue Eng. 12 (2006) 537–546.
[13] J.S. Park, K. Park, D.G. Woo, H.N. Yang, H.M. Chung, K.H. Park, PLGA microsphere construct coated with TGF-beta 3 loaded nanoparticles for neocartilage formation, Biomacromolecules 9 (2008) 2162–2169.
[14] A. Moshaverinia, S. Ansari, C.D. Chen, X.T. Xu, K. Akiyama, M.L. Snead, H.H. Zadeh, S.T. Shi, Co-encapsulation of anti-BMP2 monoclonal antibody and mesenchymal stem cells in alginate microspheres for bone tissue engineering, Biomaterials 34 (2013) 6572–6579.
[15] J.M. Curran, S. Fawcett, L. Hamilton, N.P. Rhodes, C.V. Rahman, M. Alexander, K. Shakesheff, J.A. Hunt, The osteogenic response of mesenchymal stem cells to an injectable PLGA bone regeneration system, Biomaterials 34 (2013) 9352–9364.
[16] S. Natesan, D.G. Baer, T.J. Walters, M. Babu, R.J. Christy, Adipose-derived stem cell delivery into collagen gels using chitosan microspheres, Tissue Eng. A 16 (2010) 1369–1384.
[17] A.M. Bratt-Leal, R.L. Carpenedo, M.D. Ungrin, P.W. Zandstra, T.C. McDevitt, Incorporation of biomaterials in multicellular aggregates modulates pluripotent stem cell differentiation, Biomaterials 32 (2011) 48–56.
[18] A.M. Bratt-Leal, A.H. Nguyen, K.A. Hammersmith, A. Singh, T.C. McDevitt, A microparticle approach to morphogen delivery within pluripotent stem cell aggregates, Biomaterials 34 (2013) 7227–7235.
[19] S.W. Kang, O. Jeon, B.S. Kim, Poly(lactic-co-glycolic acid) microspheres as an injectable scaffold for cartilage tissue engineering, Tissue Eng. 11 (2005) 438–447.
[20] R. Eswaramoorthy, C.C. Chang, S.C. Wu, G.J. Wang, J.K. Chang, M.L. Ho, Sustained release of PTH(1–34) from PLGA microspheres suppresses osteoarthritis progression in rats, Acta Biomater. 8 (2012) 2254–2262.
[21] E. Bible, O. Qutachi, D.Y. Chau, M.R. Alexander, K.M. Shakesheff, M. Modo, Neo-vascularization of the stroke cavity by implantation of human neural stem cells on VEGF-releasing PLGA microparticles, Biomaterials 33 (2012) 7435–7446.
[22] C.M. Coleman, C. Curtin, F.P. Barry, C. O'Flatharta, J.M. Murphy, Mesenchymal stem cells and osteoarthritis: remedy or accomplice? Hum. Gene Ther. 21 (2010) 1239–1250.
[23] A.M. Mackay, S.C. Beck, J.M. Murphy, F.P. Barry, C.O. Chichester, M.F. Pittenger, Chondrogenic differentiation of cultured human mesenchymal stem cells from marrow, Tissue Eng. 4 (1998) 415–428.
[24] B. Johnstone, M. Alini, M. Cucchiarini, G.R. Dodge, D. Eglin, F. Guilak, H. Madry, A. Mata, R.L. Mauck, C.E. Semino, M.J. Stoddart, Tissue engineering for articular cartilage repair—the state of the art, Eur. Cell Mater. 25 (2013) 248–267.
[25] K. Kawasaki, M. Ochi, Y. Uchio, N. Adachi, M. Matsusaki, Hyaluronic acid enhances proliferation and chondroitin sulfate synthesis in cultured chondrocytes embedded in collagen gels, J. Cell. Physiol. 179 (1999) 142–148.
[26] H. Zhu, N. Mitsuhashi, A. Klein, L.W. Barsky, K. Weinberg, M.L. Barr, A. Demetriou, G.D. Wu, The role of the hyaluronan receptor CD44 in mesenchymal stem cell migration in the extracellular matrix, Stem Cells 24 (2006) 928–935.
[27] G. Lisignoli, S. Cristino, A. Piacentini, C. Cavallo, A.I. Caplan, A. Facchini, Hyaluronan-based polymer scaffold modulates the expression of inflammatory and degradative factors in mesenchymal stem cells: Involvement of Cd44 and Cd54, J. Cell. Physiol. 207 (2006) 364–373.
[28] S. Browne, G. Fontana, B.J. Rodriguez, A. Pandit, A protective extracellular matrix-based gene delivery reservoir fabricated by electrostatic charge manipulation, Mol. Pharm. 9 (2012) 3099–3106.
[29] L.D. Solorio, E.L. Vieregge, C.D. Dhami, P.N. Dang, E. Alsberg, Engineered cartilage via self-assembled hMSC sheets with incorporated biodegradable gelatin microspheres releasing transforming growth factor-beta1, J. Control. Release 158 (2012) 224–232.
[30] S. Ansboro, U. Greiser, F. Barry, M. Murphy, Strategies for improved targeting of therapeutic cells: implications for tissue repair, Eur. Cell Mater. 23 (2012) 310–318(discussion 318–319).
[31] D.A. Rothenfluh, H. Bermudez, C.P. O'Neil, J.A. Hubbell, Biofunctional polymer nanoparticles for intra-articular targeting and retention in cartilage, Nat. Mater. 7 (2008) 248–254.
[32] A. Szarpak, I. Pignot-Paintrand, C. Nicolas, C. Picart, R. Auzely-Velty, Multilayer assembly of hyaluronic acid/poly(allylamine): control of the buildup for the production of hollow capsules, Langmuir 24 (2008) 9767–9774.
[33] J.M. Murphy, K. Dixon, S. Beck, D. Fabian, A. Feldman, F. Barry, Reduced chondrogenic and adipogenic activity of mesenchymal stem cells from patients with advanced osteoarthritis, Arthritis Rheum. 46 (2002) 704–713.
[34] J.M. Murphy, D.J. Fink, E.B. Hunziker, F.P. Barry, Stem Cell Therapy in a Caprine Model of Osteoarthritis, Arthritis Rheum. 48 (2003) 3464–3474.
[35] C.M. Coleman, E.E. Vaughan, D.C. Browe, E. Mooney, L. Howard, F. Barry, Growth differentiation factor-5 enhances *in vitro* mesenchymal stromal cell chondrogenesis and hypertrophy, Stem Cells Dev. 22 (2013) 1968–1976.
[36] A. Roth, J. Mollenhauer, A. Wagner, R. Fuhrmann, A. Straub, R.A. Venbrocks, P. Petrow, R. Brauer, H. Schubert, J. Ozegowski, G. Peschel, P.J. Muller, R.W. Kinne, Intra-articular injections of high-molecular-weight hyaluronic acid have biphasic effects on joint inflammation and destruction in rat antigen-induced arthritis, Arthritis Res. Ther. 7 (2005) R677–R686.
[37] Y. Kang, W. Eger, H. Koepp, J.M. Williams, K.E. Kuettner, G.A. Homandberg, Hyaluronan suppresses fibronectin fragment-mediated damage to human cartilage explant cultures by enhancing proteoglycan synthesis, J. Orthop. Res. 17 (1999) 858–869.
[38] G. D., G. S., Yu. Lvov, Assembly of Thin Films by Means of Successive Deposition of Alternate Layers of DNA and Poly(allylamine), Macromolecules 26 (1993) 5396–5399.
[39] S.J. Kim, S.R. Shin, K.B. Lee, Y.D. Park, S.I. Kim, Synthesis and characteristics of polyelectrolyte complexes composed of chitosan and hyaluronic acid, J. Appl. Polym. Sci. 91 (2004) 2908–2913.
[40] A.G. Skirtach, A.M. Yashchenok, H. Mohwald, Encapsulation, release and applications of LbL polyelectrolyte multilayer capsules, Chem. Commun. (Camb.) 47 (2011) 12736–12746.
[41] J.A. Champion, Y.K. Katare, S. Mitragotri, Particle shape: a new design parameter for micro- and nanoscale drug delivery carriers, J. Control. Release 121 (2007) 3–9.

[42] J.Y. Ko, Y.J. Choi, G.J. Jeong, G.I. Im, Sulforaphane-PLGA microspheres for the intra-articular treatment of osteoarthritis, Biomaterials 34 (2013) 5359–5368.
[43] G. Lisignoli, S. Cristino, A. Piacentini, S. Toneguzzi, F. Grassi, C. Cavallo, N. Zini, L. Solimando, N. Mario Maraldi, A. Facchini, Cellular and molecular events during chondrogenesis of human mesenchymal stromal cells grown in a three-dimensional hyaluronan based scaffold, Biomaterials 26 (2005) 5677–5686.
[44] S. Mizrahy, S.R. Raz, M. Hasgaard, H. Liu, N. Soffer-Tsur, K. Cohen, R. Dvash, D. Landsman-Milo, M.G. Bremer, S.M. Moghimi, D. Peer, Hyaluronan-coated nanoparticles: the influence of the molecular weight on CD44-hyaluronan interactions and on the immune response, J. Control. Release 156 (2011) 231–238.
[45] D. Trigkilidas, A. Anand, The effectiveness of hyaluronic acid intra-articular injections in managing osteoarthritic knee pain, Ann. R. Coll. Surg. Engl. 95 (2013) 545–551.
[46] E.N.B. Davidson, P.M. van der Kraan, W.B. van den Berg, TGF-beta and osteoarthritis, Osteoarthr. Cartil. 15 (2007) 597–604.
[47] T.I. Morales, A.B. Roberts, Transforming Growth Factor-Beta Regulates the Metabolism of Proteoglycans in Bovine Cartilage Organ-Cultures, J. Biol. Chem. 263 (1988) 12828–12831.
[48] C.H. Lee, J.L. Cook, A. Mendelson, E.K. Moioli, H. Yao, J.J. Mao, Regeneration of the articular surface of the rabbit synovial joint by cell homing: a proof of concept study, Lancet 376 (2010) 440–448.
[49] A. Mendelson, E. Frank, C. Allred, E. Jones, M. Chen, W. Zhao, J.J. Mao, Chondrogenesis by chemotactic homing of synovium, bone marrow, and adipose stem cells *in vitro*, FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol. 25 (2011) 3496–3504.
[50] M. Morille, T. Van-Thanh, X. Garric, J. Cayon, J. Coudane, D. Noel, M.C. Venier-Julienne, C.N. Montero-Menei, New PLGA-P188-PLGA matrix enhances TGF-beta3 release from pharmacologically active microcarriers and promotes chondrogenesis of mesenchymal stem cells, J. Control. Release 170 (2013) 99–110.
[51] K.L. Spiller, Y. Liu, J.L. Holloway, S.A. Maher, Y.L. Cao, W. Liu, G.D. Zhou, A.M. Lowman, A novel method for the direct fabrication of growth factor-loaded microspheres within porous nondegradable hydrogels: controlled release for cartilage tissue engineering, J. Control. Release 157 (2012) 39–45.
[52] A.H. Huang, A. Stein, R.S. Tuan, R.L. Mauck, Transient exposure to transforming growth factor beta 3 improves the mechanical properties of mesenchymal stem cell-laden cartilage constructs in a density-dependent manner, Tissue Eng. A 15 (2009) 3461–3472.
[53] B.A. Byers, R.L. Mauck, I.E. Chiang, R.S. Tuan, Transient exposure to transforming growth factor beta 3 under serum-free conditions enhances the biomechanical and biochemical maturation of tissue-engineered cartilage, Tissue Eng. A 14 (2008) 1821–1834.
[54] E.J. Caterson, L.J. Nesti, W.J. Li, K.G. Danielson, T.J. Albert, A.R. Vaccaro, R.S. Tuan, Three-dimensional cartilage formation by bone marrow-derived cells seeded in polylactide/alginate amalgam, J. Biomed. Mater. Res. 57 (2001) 394–403.
[55] M. Kim, I.E. Erickson, M. Choudhury, N. Pleshko, R.L. Mauck, Transient exposure to TGF-beta3 improves the functional chondrogenesis of MSC-laden hyaluronic acid hydrogels, J. Mech. Behav. Biomed. Mater. 11 (2012) 92–101.
[56] S.H. Kim, J. Turnbull, S. Guimond, Extracellular matrix and cell signalling: the dynamic cooperation of integrin, proteoglycan and growth factor receptor, J. Endocrinol. 209 (2011) 139–151.
[57] K.W. Kavalkovich, R.E. Boynton, J.M. Murphy, F. Barry, Chondrogenic differentiation of human mesenchymal stem cells within an alginate layer culture system, *In Vitro* Cell. Dev. Biol. Anim. 38 (2002) 457–466.
[58] S.H. Bhang, J.Y. Jeon, W.G. La, J.Y. Seong, J.W. Hwang, S.E. Ryu, B.S. Kim, Enhanced chondrogenic marker expression of human mesenchymal stem cells by interaction with both TGF-beta3 and hyaluronic acid, Biotechnol. Appl. Biochem. 58 (2011) 271–276.
[59] C. Chung, J.A. Burdick, Influence of three-dimensional hyaluronic acid microenvironments on mesenchymal stem cell chondrogenesis, Tissue Eng. A 15 (2009) 243–254.
[60] S.-C. Wu, C.-H. Chen, J.-K. Chang, Y.-C. Fu, C.-K. Wang, R. Eswaramoorthy, Y.-S. Lin, Y.-H. Wang, S.-Y. Lin, G.-J. Wang, M.-L. Ho, Hyaluronan initiates chondrogenesis mainly via CD44 in human adipose-derived stem cells, J. Appl. Physiol. 114 (2013) 1610–1618.
[61] I.D. Dinbergs, L. Brown, E.R. Edelman, Cellular response to transforming growth factor-beta1 and basic fibroblast growth factor depends on release kinetics and extracellular matrix interactions, J. Biol. Chem. 271 (1996) 29822–29829.
[62] S. Dooley, P. ten Dijke, TGF-beta in progression of liver disease, Cell Tissue Res. 347 (2012) 245–256.
[63] H.M. van Beuningen, P.M. van der Kraan, O.J. Arntz, W.B. van den Berg, Transforming growth factor-beta 1 stimulates articular chondrocyte proteoglycan synthesis and induces osteophyte formation in the murine knee joint, Lab. Invest. J. Tech. Methods Pathol. 71 (1994) 279–290.
[64] P.M. van der Kraan, W.B. van den Berg, Chondrocyte hypertrophy and osteoarthritis: role in initiation and progression of cartilage degeneration? Osteoarthr. Cartil. 20 (2012) 223–232.
[65] H.M. van Beuningen, H.L. Glansbeek, P.M. van der Kraan, W.B. van den Berg, Osteoarthritis-like changes in the murine knee joint resulting from intra-articular transforming growth factor-beta injections, Osteoarthr. Cartil. 8 (2000) 25–33.
[66] C. Bouffi, O. Thomas, C. Bony, A. Giteau, M.C. Venier-Julienne, C. Jorgensen, C. Montero-Menei, D. Noel, The role of pharmacologically active microcarriers releasing TGF-beta3 in cartilage formation *in vivo* by mesenchymal stem cells, Biomaterials 31 (2010) 6485–6493.
[67] A.B. Roberts, M.B. Sporn, R.K. Assoian, J.M. Smith, N.S. Roche, L.M. Wakefield, U.I. Heine, L.A. Liotta, V. Falanga, J.H. Kehrl, et al., Transforming growth factor type beta: rapid induction of fibrosis and angiogenesis *in vivo* and stimulation of collagen formation *in vitro*, Proc. Natl. Acad. Sci. U. S. A. 83 (1986) 4167–4171.