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## Enhanced MSC Chondrogenesis Following Delivery of TGF- $\beta$ 3 from Alginate Microspheres within Hyaluronic Acid Hydrogels *In Vitro* and *In Vivo*

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### Abstract

Mesenchymal stem cells (MSCs) are being recognized as a viable cell source for cartilage repair and members of the transforming growth factor-beta (TGF- $\beta$ ) superfamily are a key mediator of MSC chondrogenesis. While TGF- $\beta$  mediated MSC chondrogenesis is well established in *in vitro* pellet or hydrogel cultures, clinical translation will require effective delivery of TGF- $\beta$ s *in vivo*. Here, we investigated the co-encapsulation of TGF- $\beta$ 3 containing alginate microspheres with human MSCs in hyaluronic acid (HA) hydrogels towards the development of implantable constructs for cartilage repair. TGF- $\beta$ 3 encapsulated in alginate microspheres with nanofilm coatings showed significantly reduced initial burst release compared to uncoated microspheres, with release times extending up to 6 days. HA hydrogel constructs seeded with MSCs and TGF- $\beta$ 3 containing microspheres developed comparable mechanical properties and cartilage matrix content compared to constructs supplemented with TGF- $\beta$ 3 continuously in culture media, whereas constructs with TGF- $\beta$ 3 directly encapsulated in the gels without microspheres had inferior properties. When implanted subcutaneously in nude mice, constructs containing TGF- $\beta$ 3 microspheres resulted in superior cartilage matrix formation to groups without TGF- $\beta$ 3 or with TGF- $\beta$ 3 added directly to the gel. However, calcification was observed in implanted constructs after 8 weeks of subcutaneous implantation. To prevent this, the co-delivery of parathyroid hormone-related protein (PTHrP) with TGF- $\beta$ 3 in alginate microspheres was pursued, resulting in partially reduced calcification. This study demonstrates that the controlled local delivery of TGF- $\beta$ 3 is essential to neocartilage formation by MSCs and that further optimization is needed to avert the differentiation of chondrogenically induced MSCs towards a hypertrophic phenotype.

### Keywords

mesenchymal stem cells; growth factor delivery; hyaluronic acid; chondrogenesis; hypertrophy; hydrogel

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## Introduction

Mesenchymal stem cells (MSCs) have emerged as a clinically relevant cell source for regenerative medicine, especially for cartilage repair. MSCs undergo chondrogenesis and deposit a cartilage specific matrix in pellet cultures and in a variety of natural and synthetic scaffold materials in the presence of the appropriate growth factors [1, 2]. Transforming growth factor-beta (TGF- $\beta$ ) superfamily members are a key requirement for chondrogenesis of MSCs [3, 4] and studies have shown that TGF- $\beta$  stimulates chondrocyte proliferation and prevents cartilage hypertrophy [5, 6]. Previous and our preliminary *in vitro* studies also indicate that continuous exposure to TGF- $\beta$ 3 is not needed and that exposure in the first week is the most critical to the chondrogenesis of MSCs [7]. Generally, the presentation of molecules such as TGF- $\beta$ 3 is simple in an *in vitro* setting, but is more complicated in an implantable system with challenges such as tissue and scaffold diffusion, foreign body response and proteolytic activity in an *in vivo* environment. However, it is of great interest to develop therapies where MSCs and a scaffold are implanted directly into cartilage defect sites for cartilage repair. This necessitates the development of a controlled release system to provide sustained local delivery of TGF- $\beta$ 3 in order to ensure therapeutic efficacy of the implanted MSC constructs.

Previously, we showed that as a natural polymer, hyaluronic acid (HA) hydrogels provide a stable 3D environment that is conducive to the chondrogenesis of MSCs in the presence of growth factors [8, 9]. However, these studies were performed with continuous exposure to chondrogenic media (containing TGF- $\beta$ 3) *in vitro*. Meanwhile, HA has also been used as a delivery carrier for a number of growth factors including IGF, PDGF, VEGF and FGF in both *in vitro* and *in vivo* studies [10–12]. These studies demonstrated that growth factors released from HA retained their bioactivity and promoted tissue development [11, 12].

Alginate microspheres, which are ionically crosslinked in the presence of multivalent cations, have been used widely for controlled delivery of growth factors due to advantages such as biocompatibility, high encapsulation efficiency, and mild fabrication conditions [13–15]. These mild conditions include the absence of organic solvent and high shear stresses together with the absence of acidic degradation products that can induce the denaturation and loss of biological activity of encapsulated proteins. Furthermore, alginate microspheres allow for high diffusion rates of macromolecules [16], and this diffusion can be controlled with a simple one step coating procedures [17, 18].

While MSCs are able to form cartilage-like tissue *in vitro* under chondrogenic induction by TGF- $\beta$ s, transplantation to the uncontrolled *in vivo* environment commonly results in extensive calcification of the ECM [19, 20]. A recent study also reported that delivery of TGF- $\beta$ 1 with MSCs results in more subchondral trabecular bone formation in a rabbit osteochondral repair site [21]. To counter this aberrant hypertrophy, parathyroid hormone-related protein (PTHrP) has been employed to inhibit hypertrophy of chondrocytes or MSCs during chondrogenesis [22–26]. However, a recent study also showed that pretreatment of MSCs with molecules (such as PTHrP or FGF2) that suppress hypertrophy did not prevent mineralization of MSC pellets ectopically implanted in nude mice [23].

With these benefits and issues in mind, the objective of this study was to develop an alginate-based delivery vehicle for TGF- $\beta$ 3 and test its efficacy in inducing human MSC chondrogenesis and neocartilage formation within HA hydrogels in both *in vitro* and *in vivo* environments. HA hydrogels combined with alginate microspheres loaded with TGF- $\beta$ 3 forms a composite carrier which may help retain TGF- $\beta$ 3 bioactivity in the scaffold and promote chondrogenesis of MSCs when implanted.

## Material and methods

### Macromer synthesis

Methacrylated HA (MeHA) was synthesized as previously reported [27]. Briefly, methacrylic anhydride (methacrylic anhydride, 94%, FW: 154.17, Sigma) was added to a solution of 1 wt% HA (sodium hyaluronate powder, research grade, MW ~74 kDa, Lifecore) in deionized (DI) water, adjusted to a pH of 8 with 5 N NaOH, and reacted on ice for 24 h. The macromer solution was purified via dialysis (MW cutoff 6–8k) against deionized water for a minimum of 48 h with repeated changes of water. The final product was obtained by lyophilization and stored at  $-20^{\circ}\text{C}$  in powder form prior to use. The final macromer products were confirmed by  $^1\text{H}$  NMR to have a methacrylation of ~27%. Lyophilized macromers were dissolved in phosphate buffered saline (PBS) containing 0.05 wt% 2-methyl-1-[4-(hydroxyethoxy) phenyl]-2-methyl-1-propanone (I2959, Ciba) for polymerization.

### Microsphere preparation

Alginate microspheres (MS) were prepared by adapting an emulsion/external gelation protocol [15]. Briefly, alginic acid sodium salt (from brown algae, Sigma) solution was combined with TGF- $\beta$ 3 (R&D Systems, MS+T group), PTHrP (PeproTech, MS+P group), or both TGF- $\beta$ 3 and PTHrP (MS+P+T group) to achieve a final alginate concentration of 2% (w/v). The mixture was then added to olive oil dropwise while homogenizing at 500 rpm with 1% (v/v) Tween 80 as surfactant. The emulsion was mixed for 3 minutes, followed by the dropwise addition of  $\text{CaCl}_2$  solution at a concentration of 200 mM. After a further 15 minutes of crosslinking, particles were then centrifuged at 1500 g for 5 minutes, the supernatant was removed and particles were subsequently washed 3 times in 2-propanol to remove the residue oil and then washed 4 times with sterile deionized water. The particles were then air-dried and resuspended in ultrapure water. Using a method modified from Srivastava *et al.*, alginate microspheres were coated with a layer of self-assembled nanofilm consisting of poly(allylamine hydrochloride) (PAH, MW15 kDa) and poly(sodium 4-styrenesulfonate) (PSS, MW1 MDa) (Sigma, St. Louis, MO)[28].

### Characterization of in vitro release of TGF- $\beta$ 3

Uncoated (Uncoated MS) or coated (Coated MS) alginate microspheres, HA hydrogel disks encapsulated with TGF- $\beta$ 3 directly without microspheres (T in HA) and HA hydrogel disks containing encapsulated coated microspheres (Coated MS in HA) were incubated in cell culture medium (DMEM, 1% ITS+Premix, 50  $\mu\text{g}/\text{ml}$  L-proline, 0.1  $\mu\text{M}$  dexamethasone, 0.9 mM sodium pyruvate, antibiotics) at  $37^{\circ}\text{C}$  and released TGF- $\beta$ 3 was quantified with an Elisa kit (R&D Systems). Containers used for the release study were pretreated with 1% BSA solution to minimize protein absorption. Cumulative release profiles were calculated by normalizing detected TGF- $\beta$ 3 to total amount detected after 28 days of release. Fresh alginate microspheres were dissolved in sodium citrate (55 mM) solution and the encapsulation efficiency of alginate microspheres was determined by normalizing the detected TGF- $\beta$ 3 to the total amount of TGF- $\beta$ 3 used in the microsphere fabrication process.

### Sample preparation and in vitro culture

Human MSCs (Lonza) were expanded to passage 3 in growth media consisting of  $\alpha$ -MEM with 16.7% FBS and 1% pen/strep. MSCs (20 million/ml) and alginate microspheres were photoencapsulated in 1.5% MeHA hydrogel disks ( $\text{O}5$  mm, 2.6 mm thickness, Figure 1A,B) and cultured in standard media (DMEM, 1% ITS+Premix, 50  $\mu\text{g}/\text{ml}$  L-proline, 0.1  $\mu\text{M}$  dexamethasone, 0.9 mM sodium pyruvate, antibiotics) supplemented with ascorbate (50  $\mu\text{g}/\text{ml}$ ), which was changed three times per week [29]. Chondrogenic media (CM) consists of

this standard media with the addition of 10 ng/ml of TGF- $\beta$ 3. In the *in vitro* study, HA constructs were prepared with (1) blank microspheres and cultured in CM media (MS-T+CM), (2) blank microspheres and cultured in standard media (MS-T-CM), (3) TGF- $\beta$ 3-loaded microspheres (MS+T, 100 ng TGF- $\beta$ 3/disk) and cultured in standard media, or (4) direct encapsulation of TGF- $\beta$ 3 (T only, 100 ng TGF- $\beta$ 3/disk) and culture in standard media. Cell viability was assessed using the LIVE/DEAD Assay Kit (Molecular Probes) where live cells are stained green with calcein-AM and dead cells stained red with ethidium homodimer.

### Subcutaneous implantation in nude mice

In the first *in vivo* study, MSC-laden (20 million/ml) HA hydrogel constructs (n = 8 for each group) were fabricated with (1) blank microspheres (MS-T), (2) direct encapsulation of TGF- $\beta$ 3 (T only, 100 ng TGF- $\beta$ 3/disk), or (3) TGF- $\beta$ 3-loaded microspheres (MS+T, 100 ng TGF- $\beta$ 3/disk) and then implanted on the following day. Four subcutaneous pockets were prepared on the backs of male nude mice (NCRNU, age 4 weeks; Taconic) and received implants. In the follow-up *in vivo* study, MSC-laden (20 million/ml) HA hydrogel disks (n = 8 for each group) were fabricated with (1) TGF- $\beta$ 3-loaded microspheres (MS+T, 100 ng TGF- $\beta$ 3/disk), (2) PTHrP-loaded microspheres (MS+P, 50 ng PTHrP/disk), or (3) TGF- $\beta$ 3+PTHrP-loaded microspheres (MS+T+P, 100 ng TGF- $\beta$ 3 and 50 ng PTHrP/disk) and then implanted on the following day. Samples were harvested 4 or 8 weeks later. Guidelines from Institutional Animal Care and Use Committee at the University of Pennsylvania were followed during all animal procedures.

### Mechanical testing

At set time points, samples were removed from the culture or retrieved from the subcutaneous space of mouse and the bulk mechanical properties of construct disks were evaluated using a custom table top testing device as described previously [30]. Briefly, samples were first equilibrated in creep to a tare load of 2 grams by an impermeable loading platen in a loading chamber filled with phosphate buffered saline. From this offset, stress relaxation tests were performed with a single compression ramp at a speed of 10%/min until reaching 10% strain. The equilibrium Young's modulus ( $E_Y$ ) was determined by the equilibrium load obtained after 1000 sec of relaxation under unconfined compression at 10% strain. The Young's moduli of the tested samples were considered undetectable if the equilibrium load at the end of stress relaxation was lower than the initial tare load, and these samples were plotted as having a Young's modulus of zero.

### Gene expression analysis

For gene expression analysis, samples were homogenized in Trizol Reagent (Invitrogen) with a tissue grinder, RNA was extracted according to the manufacturer's instructions, and the RNA concentration was determined using an ND-1000 spectrophotometer (Nanodrop Technologies). One microgram of RNA from each sample was reverse transcribed into cDNA using reverse transcriptase (Superscript II, Invitrogen) and oligoDT (Invitrogen). Polymerase chain reaction (PCR) was performed on an Applied Biosystems 7300 Real-Time PCR system using Taqman primers and probes specific for GAPDH (housekeeping gene) and other genes of interest. Sequences of the primers and probes used are listed in Table 1. The relative gene expression was calculated using the  $\Delta\Delta C_T$  method, where fold difference was calculated using the expression  $2^{\Delta\Delta C_T}$ . Each sample was internally normalized to GAPDH, and each group was normalized to the expression levels of MSCs at the time of encapsulation (i.e., after expansion and before differentiation). Relative expression levels greater than 1 represent up-regulation with culture, while relative expression levels less than 1 represent down-regulation of that gene compared to that of initially encapsulated MSCs.

## Biochemical analysis

One-half of each construct was weighed wet, lyophilized, reweighed dry, and digested in 0.5 mg/ml Proteinase-K (Fisher Scientific) at 56°C for 16 hrs. The PicoGreen assay (Invitrogen, Molecular Probes) was used to quantify the DNA content of the constructs with Lambda phage DNA (0–1 mg/ml) as a standard [31]. For each sample, both the mass of the entire gel and the half gel used for DNA assay were measured. The total amount of DNA per sample was calculated by scaling the amount of DNA detected in the half gel by a weight ratio (total weight/half weight). The GAG content was measured using the dimethylmethylene blue (DMMB, Sigma Chemicals) dye-binding assay with shark chondroitin sulfate (0–50 mg/ml) as a standard [32]. The overall collagen content was assessed by measuring the orthohydroxyproline (OHP) content via dimethylaminobenzaldehyde and chloramine T assay. Collagen content was calculated by assuming a 1:7.5 OHP-to-collagen mass ratio [33]. The collagen and GAG contents were normalized to the disk wet weight.

## Histological analysis

The remaining halves of the constructs were fixed in 4% formalin for 24 h, embedded in paraffin, and processed using standard histological procedures. The histological sections (8  $\mu$ m thick) were stained for targets of interest using the Vectastain ABC kit and the DAB Substrate kit for peroxidase (Vector Labs). Briefly, sections were predigested in 0.5 mg/ml hyaluronidase for 30 min at 37°C and incubated in 0.5 N acetic acid for 4 h at 4°C to swell the samples prior to overnight incubation with primary antibodies at dilutions of 1:100, 1:200, and 1:3 for chondroitin sulfate (mouse monoclonal anti-chondroitin sulfate, Sigma), and type I (mouse monoclonal anti-collagen type I, Sigma) and type II collagen antibodies (mouse monoclonal anti-collagen type II, Developmental Studies Hybridoma Bank), respectively. Non-immune controls underwent the same procedure without primary antibody incubation.

## Statistical analysis

All data are presented as mean  $\pm$  standard deviation. Statistica (Statsoft, Tulsa, OK) was used to perform statistical analyses using two-way ANOVA and the Tukey HSD post hoc test of the means ( $n=4$  samples per group) with culture duration and experimental groups as independent factors.

## Results

### Microsphere fabrication and release behavior

Alginate microspheres were fabricated using an emulsion process followed by coating. The coating process did not significantly change the size of the alginate microspheres, which had a mean diameter of  $38.9 \pm 9.2 \mu\text{m}$ . Alginate microspheres with nanofilm coating exhibited improved retention of the encapsulated BSA-FITC molecules compared to the uncoated bare microspheres (Figure 1C). The encapsulation efficiency of TGF- $\beta$ 3 in the uncoated and coated microspheres was  $79.6 \pm 5.6\%$  and  $85.4 \pm 9.5\%$ , respectively. Furthermore, when TGF- $\beta$ 3 was encapsulated, the nanofilm coating reduced the initial burst release of the TGF- $\beta$ 3 from alginate microspheres in the first 2 days (Coated MS vs. Uncoated MS, Figure 2A, B). When TGF- $\beta$ 3 was directly encapsulated in acellular 1.5% (w/v) HA hydrogels without alginate microspheres, 68% of the total amount of TGF- $\beta$ 3 encapsulated was released after 1 day and the release profile plateaued after day 2 (T in HA, Figure 2A,B). By encapsulating coated alginate microspheres loaded with TGF- $\beta$ 3 into the HA hydrogel, the lowest initial burst release of 37% after 1 day was achieved and the TGF- $\beta$ 3 released remained lower than the “T in HA” group up to day 3 (Figure 2A,B).



### ***In vitro* neocartilage formation**

To evaluate the efficacy of released TGF- $\beta$ 3 in promoting chondrogenesis, human MSCs in HA hydrogels were assessed through day 28. Cells in all groups that received TGF- $\beta$ 3 remained nearly completely viable (MS+T, MS-T+CM and T only). However, there was a significant reduction in the number of viable cells (~ 90% decrease compared to “MS-T+CM” as estimated using Image J) in the absence of TGF- $\beta$ 3 in medium on day 28 (MS-T-CM, Figure 3).

On day 7, all groups supplemented with TGF- $\beta$ 3 in the medium (MS-T+CM), in the HA hydrogel (T in only), or via microspheres (MS+T) exhibited similar degrees of upregulation in type II collagen expression. The expression of type I collagen remained unchanged from that of the pre-encapsulation MSCs in the presence of TGF- $\beta$ 3 (Figure 4). The “negative control” group (MS-T-CM), which received no TGF- $\beta$ 3 supplementation in medium, showed significantly lower collagen II and aggrecan expression and much higher collagen I expression compared to all other groups (Figure 4). Interestingly, the groups that were chondrogenically induced with a single initial supplementation of TGF- $\beta$ 3 loaded in alginate microspheres (MS+T) had lower collagen X expression than the “MS-T+CM” group which received a continuous supply of TGF- $\beta$ 3 in medium.

Tissue engineered cartilage disks that received a single initial dosage of TGF- $\beta$ 3 encapsulated in nanofilm coated microspheres (MS+T) developed similar levels of mechanical stiffness, collagen and GAG content compared to the “positive control” (MS-T+CM) which received continuous TGF- $\beta$ 3 supplementation in medium, whereas these values for the group containing directly encapsulated TGF- $\beta$ 3 without microspheres (T only) were all lower than the “MS-T+CM” group (Figure 5A-C). The “negative control” group (MS-T-CM) showed minimal cartilage matrix production and decreasing cell viability, as indicated by viability staining (Figure 3) and DNA content in the absence of TGF- $\beta$ 3 (Figure 5A-D).

Immunohistochemical staining for type II collagen and chondroitin sulfate showed intense and distributed staining in all groups supplemented with TGF- $\beta$ 3 either in medium or via microspheres (MS-T+CM, T only, MS+T) on day 28, whereas the staining for type I collagen was low (Figure 6). There was only isolated matrix staining present in the negative control group (MS-T-CM) (Figure 6).

### ***In vivo* neocartilage formation**

To test the *in vivo* efficacy of this delivery system, HA hydrogel disks were implanted in subcutaneous pockets of nude mice. After 56 days, out of 8 samples implanted per group, 1, 3 and 5 samples were recovered from the “MS-T”, “T only” and “MS+T” groups, respectively. The implants that contained coated alginate microspheres loaded with TGF- $\beta$ 3 (MS+T) were opaque and white and were palpably stiffer than implants that were loaded with TGF- $\beta$ 3 directly without alginate microspheres (T only) (which were translucent and soft) (Figure 7A). Only one intact implant was recovered from the group that did not have TGF- $\beta$ 3 encapsulated (MS-T). The implants recovered from this group were nearly transparent and very soft (Figure 7A). Furthermore, the “MS+T” groups developed significantly higher GAG, Collagen and DNA contents and implant wet weights compared to the “T only” group after 56 days of implantation (Figure 7B-D). The “MS+T” group had intense and distributed staining for type II collagen and chondroitin sulfate but faint staining for type I collagen (Figure 7E). In comparison, the staining of these markers in the “T only” group was faint and mostly localized in the pericellular region (Figure 7E). However, the “MS+T” group exhibited a significant increase in mechanical stiffness and calcium content

accompanied by a decrease in water content of the implanted constructs (Figure 8AC). Von Kossa staining indicated extensive calcification in the “MS+T” constructs (Figure 8D).

In a follow-up *in vivo* study the impact of PTHrP inclusion was examined after 56 days of implantation. *In vitro* release profile showed significant burst release of PTHrP out of alginate microspheres in the HA hydrogels (Figure 9A). Disks containing alginate microspheres loaded with only TGF- $\beta$ 3 (“MS+T”) or both TGF- $\beta$ 3 and PTHrP (“MS+P+T”) exhibited a dramatic increase in mechanical stiffness from day 28 (Figure 9B). The disks containing only PTHrP (“MS+P”) were translucent and soft with non-detectable mechanical stiffness (not shown). However, Von Kossa staining indicated extensive calcification in both “MS+T” and “MS+P+T” constructs on both day 28 and day 56 (Figure 9C). While cartilage matrix content of the “MS+P” group remained low, the “MS+P+T” group developed slightly higher GAG content and similar total collagen content compared to the “MS+T” group (Figure 9D,E). Importantly, calcium content of the “MS+P+T” group was moderately reduced compared to the “MS+T” group on day 56 (Figure 9F). The “MS+P” group had significantly less calcium content and Von Kossa staining than the other two groups (Figure 9C,F).

## Discussion

HA scaffolds are finding application as cell carriers in the repair of cartilage defects. Recent *in vitro* studies demonstrated successful chondrogenesis of MSCs photoencapsulated in HA hydrogels formed through the crosslinking of HA modified with methacrylate groups [8, 9, 34]. To improve the translation of these materials to *in vivo* applications, it is of great importance to develop a delivery vehicle capable of controlled local TGF- $\beta$ 3 release for the induction of MSC chondrogenesis. This is because applications of cartilage tissue engineering and regeneration using MSCs may be simplified by eliminating the step of *in vitro* culture of tissue engineered cartilage prior to implantation by directly implanting MSCs into defects. Several previous studies have showed that TGF- $\beta$ 1 delivered via microspheres enhanced articular chondrocytes proliferation and cartilage matrix deposition in hydrogel scaffolds *in vitro* [35, 36]. Recent studies also suggested that TGF-loaded microspheres embedded in hydrogel scaffold resulted in significant upregulation of chondrogenic gene expression by MSCs *in vitro* [37, 38]. Another recent study demonstrated that TGF- $\beta$ 3 delivered in collagen gel enhanced regeneration of articular cartilage in rabbit humeral joints [39]. In this study we demonstrated that an early dose of TGF- $\beta$ 3 delivered via a composite carrier consisting of nanofilm-coated alginate microspheres and HA hydrogel was sufficient to support human MSC chondrogenesis and neocartilage formation. This sustained delivery of TGF- $\beta$ 3 resulted in enhanced expression of chondrogenic genes and higher levels of cartilage specific matrix deposition by human MSCs in HA hydrogels compared to uncoated microspheres or a bolus encapsulation of TGF- $\beta$ 3 *in vitro*. The following *in vivo* study further showed that this TGF- $\beta$ 3 delivery is essential to neocartilage formation *in vivo* compared to a bolus encapsulation of TGF- $\beta$ 3.

Alginate gels have a wide pore size distribution due to the open lattice structure of the matrix [40], resulting in enzyme release from these matrices. Low molecular weight encapsulants (<20 kDa) diffuse freely in and out of the beads, and this diffusion from the alginate beads was found to be unaffected by the concentration of alginate or CaCl<sub>2</sub> [41, 42]. The homodimeric recombinant human TGF- $\beta$ 3 has a predicted molecular mass of approximately 25 kDa. In this study most of the TGF- $\beta$ 3 encapsulated diffused out of bare alginate microspheres quickly within 2 days. The ionic nature of the molecule, i.e., cationic, anionic, and nonionic also influences the release behavior of drugs from alginate beads [43]. Recombinant human TGF- $\beta$ 3 used in this study is anionic at pH 7.2 (pI = 6.1) and is expected to be repelled by the negatively charged alginate matrix, resulting in faster release.

Therefore, a self-assembled bilayer nanofilm coating made of polyelectrolytes of opposing charges was used to reduce the initial burst release of TGF- $\beta$ 3 from the alginate microspheres. One advantage of this nanofilm coating as a diffusion barrier is that its permeability can be tuned by adjusting the number of bilayers [28]. In this study, one bilayer of coating was sufficient to extend the release of TGF- $\beta$ 3 from alginate microspheres to a timeframe of around 4–5 days that is deemed necessary for MSC chondrogenesis. Multiple bilayers of coating could be used in future studies to further slow down the release of encapsulated molecules that are needed at later times.

It has been shown that compared with continuous supplementation, an initial transient exposure of TGF- $\beta$ 3 for 2 weeks enhanced the maturation of tissue engineered cartilage seeded with bovine chondrocytes and MSCs *in vitro* [29, 44]. From a molecular biology perspective, though TGF- $\beta$ 3 may be imperative for initiation of upregulation of sox9 expression (a crucial chondrogenic gene) it is not required for continued expression of chondrogenic markers such as type II collagen at later stages of chondrogenesis [45, 46]. Our recent preliminary studies also showed that while a brief exposure to TGF- $\beta$ 3 for less than 2 days is also less than optimal, supply of a high dosage of TGF- $\beta$ 3 (100 ng/ml) only in the first week of *in vitro* culture led to a similar amount of cartilage matrix accumulation in human MSC-seeded hydrogel constructs by week 9 compared to constructs receiving continuous TGF- $\beta$ 3 supplementation of regular dosage (10 ng/ml) (unpublished data). In this study, when TGF- $\beta$ 3 was encapsulated in the coated microspheres first and then encapsulated in the HA hydrogels (Coated MS in HA) the retention of TGF- $\beta$ 3 in the hydrogel was significantly improved compared to direct encapsulation of TGF- $\beta$ 3 in HA hydrogels (T in HA). When an identical amount of TGF- $\beta$ 3 was delivery either via coated microspheres (MS+T) or direct encapsulation without using microspheres (T only) into the MSC-laden HA hydrogel constructs, microsphere delivery resulted in significantly higher cartilage matrix content than direct encapsulation under *in vitro* or *in vivo* condition. This finding indicated that extending the exposure of TGF- $\beta$ 3 to about 6 days as seen in the “MS +T” group is essential to neocartilage formation by MSCs under both *in vitro* and *in vivo* conditions.

In this study, significant calcification was observed in the HA hydrogel constructs after 8 weeks of subcutaneous implantation in nude mice with only an initial administration of TGF- $\beta$ 3 via alginate microspheres. Co-delivering PTHrP with TGF- $\beta$ 3 using alginate microspheres resulted in only a modest reduction in calcium content on day 56 and failed to prevent extensive mineralization. This is likely due to the quick release of PTHrP, which has a smaller molecular weight (9.8 kD) than TGF- $\beta$ 3 (25 kD), within the first week similar to the release of TGF- $\beta$ 3 (Figure 9A). The effect of PTHrP on MSC hypertrophy inhibition *in vivo* might be optimized if its release from alginate microspheres can be delayed to the second week of implantation when MSCs begin to express hypertrophic markers [19]. This can be achieved by adding additional layers of nanofilm coatings, which has been shown to further slow down the release of encapsulated molecules compared to a single layer of coating [28]. Future studies should be focused on developing strategies to stabilize the chondrogenic phenotype of MSCs *in vivo*. One potential direction is to develop controlled release vehicles capable of delayed delivery of hypertrophy inhibiting molecules. It was also noted that though no blood vessels were found inside the implanted hydrogels, they were usually covered by a highly vascularized fibrous capsule (Figure 7A). It has been reported that hypertrophic chondrocytes secrete vascular endothelial growth factor (VEGF) and vascularization is closely involved in the replacement of cartilage by bone during endochondral ossification [47]. A recent study employed suramin, an anti-angiogenic agent, to suppress hypertrophy of engineered cartilage seeded with periosteal cells *in vivo* [48]. Therefore, molecules capable of suppressing vascularization can also be incorporated in the alginate microspheres to prevent mineralization of MSCs ectopically. In addition, other



therapeutic molecules involved in skeletal tissue remodeling can also be explored for potential application in regulating MSC hypertrophy. A recent study showed that pretreatment of MSCs with retinoid agonists substantially inhibited intramuscular and subcutaneous heterotopic ossification irreversibly after implantation in mice [49]. Lastly, previous studies demonstrated that articular chondrocytes secrete hypertrophy inhibition molecules such as PTHrP, FGF-2 and TGF- $\beta$ s [26, 45]. A previous study by our group also showed that coculture of MSCs and a small fraction of articular chondrocytes in HA hydrogels not only enhanced chondrogenesis of MSCs but also reduced the expression of hypertrophic markers by MSCs [50]. Therefore, another potential strategy is to seed the MSC-laden constructs with articular chondrocytes that will serve as “living” drug delivery vehicles to supply hypertrophy regulating molecules continuously.

## Conclusions

Our findings demonstrated that a single administration of TGF- $\beta$ 3 in nanofilm coated alginate microspheres encapsulated in HA hydrogels promoted chondrogenesis of encapsulated MSCs and resulted in superior neocartilage formation compared to a bolus direct encapsulation of TGF- $\beta$ 3 under both *in vitro* and *in vivo* conditions. This composite TGF- $\beta$ 3 delivery system will potentially simplify the proposed autologous MSC therapy for cartilage repair substantially by eliminating the step of *in vitro* culture, allowing direct implantation of MSCs into defect sites. However, future effort is needed to stabilize the chondrogenic phenotype and arrest hypertrophic differentiation of the implanted MSCs.

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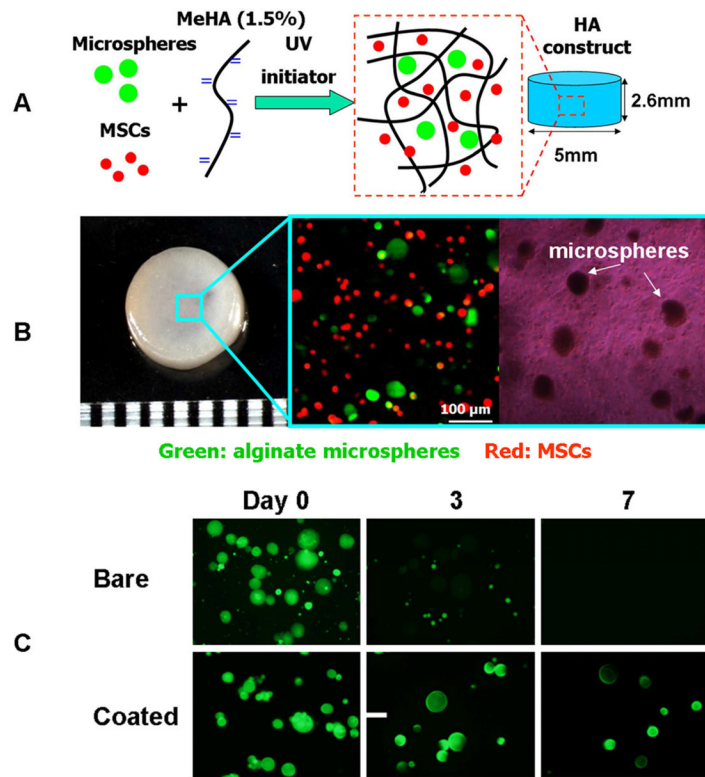
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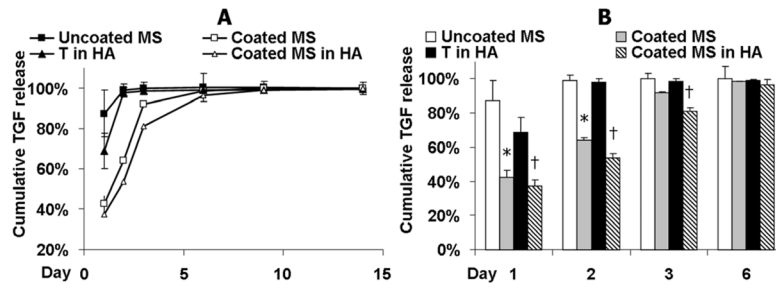
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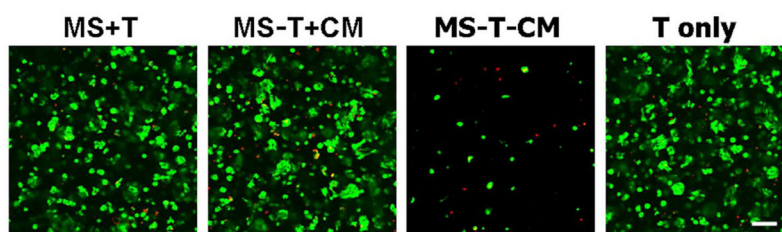
**Figure 1.** Photoencapsulation of alginate microspheres (4 mg/ml) and MSCs (20 million/ml) into methacrylated HA (MeHA) hydrogel disks (A); fabricated HA hydrogel disk and fluorescent and bright field microscopic images of MSCs (membrane labeled with red dye) and alginate microspheres (containing FITC-labeled protein) encapsulated in HA gels (B); release of encapsulated BSA-FITC from bare and coated alginate microspheres over 7 days in PBS (C), scale bar = 50 μm.



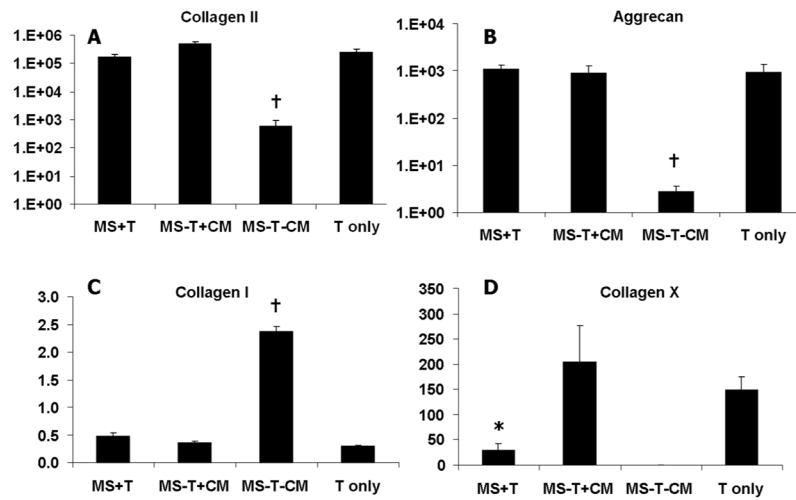


**Figure 2.**

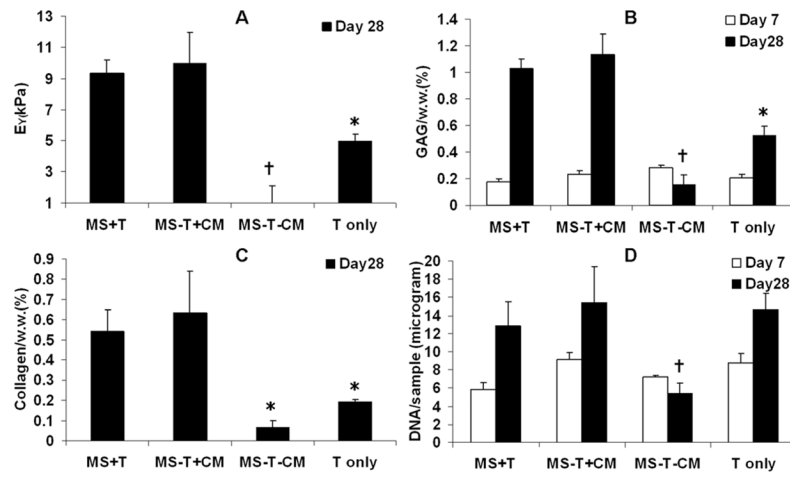
Release profile over 14 days (A) and 6 days (B) of TGF- $\beta$ 3 encapsulated in bare microspheres (Uncoated MS); coated microspheres (Coated MS); coated microspheres and further photoencapsulated in HA hydrogel (Coated MS in HA) and TGF- $\beta$ 3 directly encapsulated in HA hydrogel (T in HA). Cumulative release is calculated based on the total amount of TGF- $\beta$ 3 detected after 28 days. \* $p < 0.05$  vs. “Uncoated MS” at the same timepoints, † $p < 0.05$  vs. “T in HA” (n=3).



**Figure 3.** Viability staining of MSCs in HA hydrogel constructs after 28 days of *in vitro* culture for groups containing microspheres with TGF- $\beta$ 3 (MS+T), containing empty microspheres either in chondrogenic media (MS-T+CM) or in standard growth media (MS-T-CM), or with TGF $\beta$ -3 alone (T only). Green: live cells; Red: dead cells; scale bar = 100  $\mu$ m.

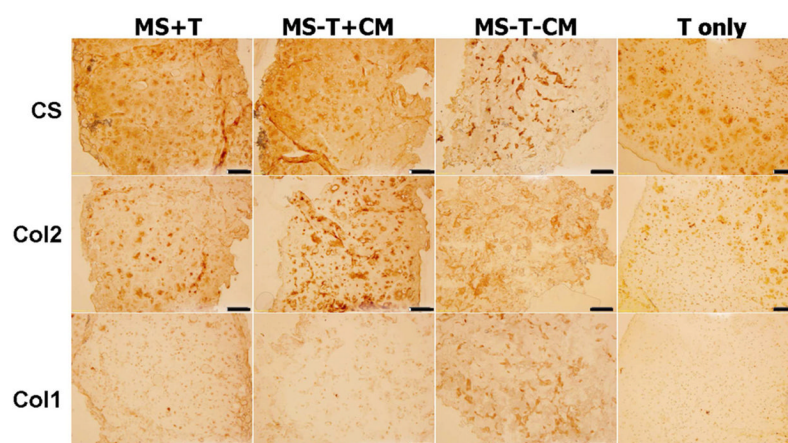


**Figure 4.** Gene expression (in fold changes, first internally normalized to GAPDH and then normalized to monolayer cells prior to encapsulation) of selected chondrogenic and hypertrophic markers in MSC-laden HA hydrogel constructs after 7 days of *in vitro* culture. \* $p < 0.05$  vs. MS-T+CM at the same culture time; † $p < 0.05$  vs. all other groups at the same culture time (n=4).



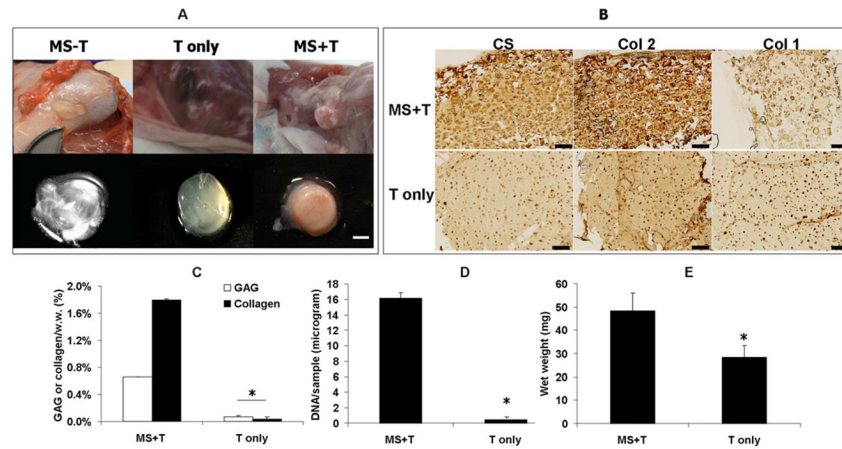
**Figure 5.**

Young's modulus (A), total collagen content normalized by sample wet weight (B), GAG content normalized by sample wet weight (C) and DNA content per sample (D) of MSC-laden HA hydrogel constructs after 7 or 28 days of *in vitro* culture (Young's modulus and collagen content were not evaluated on day 7 due to low values). \* $p < 0.05$  vs. MS-T+CM at the same culture time; † $p < 0.05$  vs. all other groups at the same culture time (n=4).



**Figure 6.** Immunohistochemical staining for chondroitin sulfate (CS), type II (Col 2) and type I (Col 1) collagen on paraffin sections of MSC-laden HA hydrogel constructs after 28 days of *in vitro* culture, scale bar = 100  $\mu$ m.

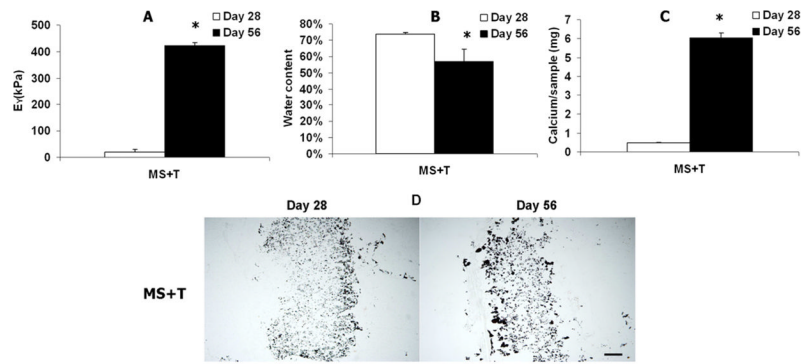




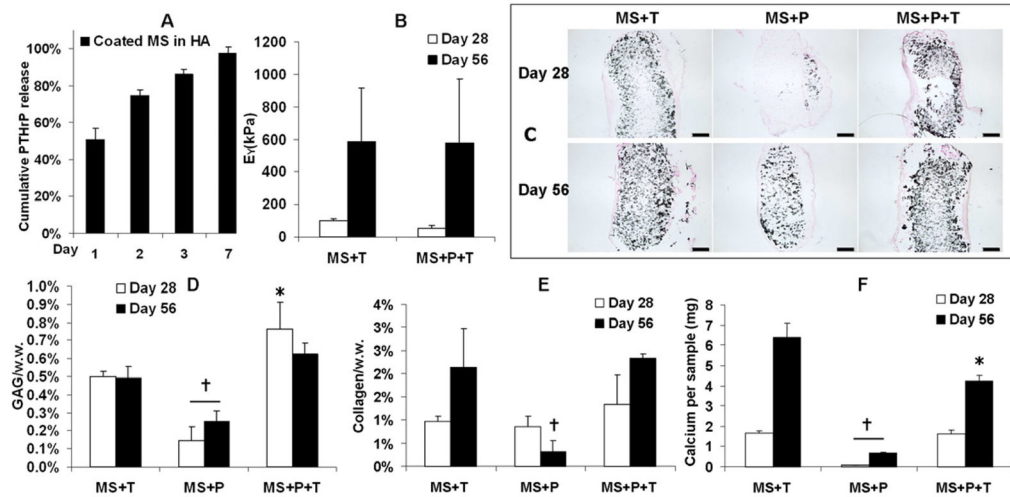
**Figure 7.**

Harvested MSC-laden HA hydrogel implants after 56 days of subcutaneous implantation in nude mice. Out of 8 samples implanted per group, 1, 3 and 5 samples were recovered from the “MS-T”, “T only” and “MS+T” groups, respectively, scale bar = 2 mm (A).

Immunohistochemical staining for chondroitin sulfate (CS), type II (Col 2) and type I (Col 1) collagen on paraffin sections of MSC-laden HA hydrogel constructs after 56 days of implantation, scale bar = 200  $\mu$ m (B). GAG and total collagen content (C), DNA content (D) and wet weight (E) of the “T only” and “MS+T” group after 56 days of implantation. (“MS-T” was not presented due to limited number of samples recovered, \* $p < 0.05$  vs. “T only” ( $n = 3 \sim 5$ )).



**Figure 8.** Young's modulus (A), water content (B) and calcium content (C) of the implanted HA hydrogel constructs containing TGF- $\beta$ 3-loaded microspheres (MS+T) after 28 or 56 days of subcutaneous implantation in nude mice, \* $p < 0.05$  vs. Day 28 ( $n = 4$ ). Von Kossa staining of the implanted HA constructs (MS+T), bar = 500  $\mu$ m (D).



**Figure 9.** Release profile of PTHrP encapsulated in coated microspheres and further photoencapsulated in HA hydrogel (Coated MS in HA) (A). Cumulative release is calculated based on the total amount of PTHrP detected. Young’s modulus (B), Von Kossa staining (C, bar = 500  $\mu$ m), GAG content normalized by sample wet weight (D), total collagen content normalized by sample wet weight (E) and calcium content per sample (F) of the implanted MSC-laden HA hydrogel constructs after 28 or 56 days of subcutaneous implantation in nude mice (Young’s modulus of the “MS+P” was not detectable due to softness), \* $p < 0.05$  vs. “MS+T, † $p < 0.05$  vs. all other groups at the same culture time (n=4).

**Table 1**

Sequences of primers and probes used for Real-Time PCR. Sequences related to gene type X collagen are proprietary to Applied Biosystems Inc. and not disclosed.

Gene	Forward Primer	Reverse Primer	Probe
GAPDH	AGGGCTGCTTTAACTCTGGTAAA	GAATTTGCCATGGGTGGAAT	CCTCAACTACATGGTTTAC
COL I	AGGACAAGAGGCATGTCTGGTT	GGACATCAGGCGCAGGAA	TTCCAGTTCGAGTATGGC
COL II	GGCAATAGCAGGTTACGTACA	CGATAACAGTCTTGCCCCACTT	CTGCACGAAACATAC
Aggrecan	TCGAGGACAGCGAGGCC	TCGAGGGTGTAGCGTGTAGAGA	ATGGAACACGATGCCTTTCACCACGA