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Site-directed differentiation of human adipose derived mesenchymal stem cells to nucleus pulposus cells using an injectable hydroxyl-functional diblock copolymer worm gel

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Abstract. Adipose-derived mesenchymal stem cells (ASCs) have been identified for their promising therapeutic potential to regenerate and repopulate the degenerate intervertebral disk (IVD), which is a major cause of lower back pain. The optimal cell delivery system remains elusive but encapsulation of cells within scaffolds is likely to offer a decisive advantage over the delivery of cells in solution by ensuring successful retention within the tissue. Herein we evaluate the use of a fully synthetic, thermoresponsive poly(glycerol monomethacrylate)-poly(2-hydroxypropyl methacrylate) (PGMA-PHPMA) diblock copolymer worm gel that mimics the structure of hydrophilic glycosaminoglycans. The objective was to use this gel to direct differentiation of human ASCs towards a nucleus pulposus (NP) phenotype, with or without the addition of discogenic growth factors TGFB or GDF-6. Accordingly, human ASCs were incorporated into a cold, free-flowing aqueous dispersion of the diblock copolymer, gelation induced by warming to 37 ◦C and cell culture was conducted for 14 days with or without growth factors to assess the expression of characteristic NP markers compared to those produced when using collagen gels. The shear-thinning nature of the biocompatible worm gel enabled encapsulated human ASCs to be injected into the IVD using a 21G needle. Moreover, we find significantly higher gene expression levels of ACAN, SOX9, KRT8 and KR18 for ASCs encapsulated within worm gels compared to collagen scaffolds, regardless of the growth factors employed. In summary, such wholly synthetic worm gels offer considerable potential as an injectable cell delivery scaffold for the treatment of degenerate disk disease by promoting the transition of ASCs towards an NP phenotype.

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Introduction

Over the past two decades, hydrogels have been extensively examined for cell biology [1-6] and tissue engineering [7-10] applications. In particular, a wide range of injectable synthetic and naturally-derived hydrogels have been designed to target nucleus pulposus (NP) cell-based therapies[10-23].

Regenerative therapies targeting the degenerate intervertebral disk (IVD) are at the forefront of research within the spinal field. In particular, considerable attention has been focusing on the development of a biological therapeutic that addresses the underlying pathogenesis of degenerative disk disease. Lower back pain (LBP) affects 80% of the UK population, with many of these cases being associated with IVD degeneration. LBP has a huge global socioeconomic impact; it is estimated to cost more than £12 billion per annum in the UK alone[24, 25].

IVDs are located between the bony vertebrae of the spinal column and are composed of three anatomically distinct regions, with the gelatinous NP - which is constrained by thick layers of radially-aligned collagen fibres - forming the annulus fibrosus (AF). A thin layer of hyaline cartilage borders the vertebrae and the disk; this is known as the cartilaginous end plates (CEPs). The highly specialized composition of the matrix within the IVD enables movement and confers resilience to compressive forces and loads[26]. Degenerative changes occur predominantly in the highly hydrophilic NP, which is composed of aggrecan (ACAN) and collagen type II (COL2). During degeneration, the extracellular matrix (ECM) of the NP is subject to high levels of degradation, in particular the loss of aggrecan. These changes result in dehydration of the ECM, which influences the biomechanical properties of the disk, ultimately leading to compromised structural and functional properties.

Current therapies for IVD degeneration include conservative symptomatic pain relief or end-stage surgery[27-32]. However, such treatments are often ineffective and merely treat the symptoms rather than the underlying pathological problem. There is currently an unmet clinical need for novel cell-based therapies that focus on repairing the damaged tissue, ultimately alleviating patient's symptoms through regenerative medicine. Many research groups have discussed the potential use of mesenchymal stem cells (MSCs) for the repair and regeneration of the damaged IVD, either alone or in combination with NP cells of the disk, as well as the use of biocompatible scaffolds and carriers[33-35].

MSCs are an attractive choice for NP regeneration for many reasons. In particular, they possess the ability to differentiate and produce matrices that are comparable to the native NP. Native adult NP cells are conventionally described as "chondrocyte-like" and characterized by their rounded morphology and expression of classic chondrogenic markers *SOX-9*, COL2A and ACAN[36]. However, the composition of the NP ECM significantly differs from that of articular cartilage, with NP tissue being substantially more proteoglycan-rich

than cartilage[37, 38]. Our own studies[39, 40], and those of others[41, 42], have identified a panel of NP-specific markers that should be taken into consideration when determining differentiation of MSCs toward an NP-phenotype for regenerative therapies. Such differentiation is often achieved using a 3D culture environment in combination with a chondrogenic medium containing transforming growth factor beta-3 (TGFβ3). However, we have previously demonstrated the potential use of other members of the TGF superfamily to induce MSC differentiation toward an NP-phenotype in MSCs derived from bone marrow and adipose tissue. Growth differentiation factor 5 (GDF/BMP-14/CDMP-1) and growth differentiation factor 6 (GDF6/BMP-13/CDMP-2) are associated with skeletal development and can be expressed by human NP cells *in vitro*[43]. Moreover, in principle they can induce NP differentiation by increasing gene expression levels of ACAN and COL2[44]. Clarke *et al.* reported using GDF6 to induce differentiation of ASCs toward an NP phenotype at both gene and protein levels. The ensuing positive benefit was comparable to that obtained via TGFβ3 induced differentiation[45].

There has been a vast amount of research focusing on the use of natural and synthetic scaffolds for regeneration. For example, recent advances in polymer scaffolds have demonstrated their capacity to restore disk biomechanics owing to their strength[42]. The use of growth factors to promote repair and regeneration of the IVD is also becoming more attractive, particularly in combination with cell-based therapies[46]. Preliminary phase I and II clinical trials utilizing MSCs are being conducted worldwide and have established the safety and efficacy of both allogeneic and autologous stem cells for use in IVD degeneration[47, 48]. Thus we now need to concentrate on optimizing the cell source and delivery of these cells to ensure successful clinical outcomes.

Previously, we reported the design, synthesis and behavior of a wholly synthetic poly(glycerol monomethacrylate)-poly(2-hydroxypropyl methacrylate) (PGMA-PHPMA) diblock copolymer worm gel that undergoes reversible degelation via a worm-to-sphere transition on cooling from 37 to 5 °C[49, 50]. Such thermoreversible behavior is important because it enables convenient sterilization via cold ultrafiltration. The rather soft[51] worm gels that are formed at 20-37 °C are the result of multiple inter-worm contacts, which effectively arrest translational diffusion[52]. These relatively weak inter-worm interactions confer shear-thinning behavior, which means that such hydrogels are readily injectable (see Figure S1). PGMA is a hydroxyl-rich polymer that is known to be both biocompatible and to minimize cell adhesion[53]. $PGMA_{63}$-$PHPMA_{135}$ worm gels can be readily synthesized directly in water and redispersed from freeze-dried powder into the desired physiological buffer (e.g. PBS) or cell culture medium to form reconstituted worm gels with essentially the same physical properties as the original worm gel[54]. Remarkably, such worm gels can also induce stasis in naïve pluripotent human stem cells[53].

Such diblock copolymer worm gels exhibit high degrees of hydration and are rich in hydroxyl functionality. Moreover, their structure resembles key components of the ECM of the gelatinous NP, particularly the highly hydrophilic glycosaminoglycans (GAGs). Thus we

decided to examine the extent to which PGMA-PHPMA worm gels can mimic components of the NP ECM. In particular, we explore whether encapsulating human ASCs within such wholly synthetic worm gels offers any benefit for differentiation toward an NP phenotype compared to collagen gels, which are widely used for various cell biology studies.

More specifically, the aim of this study is to elucidate the potential use of such wholly synthetic worm gels to direct differentiation of human ASCs towards an NP-phenotype, with or without the addition of chondrogenic/discogenic growth factors.

Experimental

Materials and methods

Mesenchymal stem cell culture

National Research Ethics Service and University of Manchester approvals were obtained prior to performing all experiments. Samples of subcutaneous adipose tissue were obtained from three female donors (mean age 43 years; ranging from 27 to 52 years) who each provided written informed consent prior to hip replacement surgery.

Adipose tissue was finely minced using a scalpel and ASCs were isolated enzymatically as previously reported[55]. ASCs were maintained at 37 °C in a humid atmosphere containing 5% v/v CO_2; standard culture medium (Minimum Essential Medium α-modification (αMEM) (Sigma-Aldrich) comprising non-essential amino acids, 110 mg dm^{-3} sodium pyruvate, 1000 mg dm^{-3} glucose supplemented with final concentrations of 100 mg dm^{-3} streptomycin, 100 U/ml penicillin, 0.25 mg dm^{-3} amphotericin, 2 mM GlutaMAX (Life Technologies) and 20% (v/v) foetal bovine serum (FBS)) was changed after five days and cells were cultured to ~80% confluence in αMEM as above supplemented with 10% FBS.

Flow cytometry was used to analyze the CD profile of ASCs and their multipotentiality was assessed along the three mesenchymal lineages using standard methods. Cells at passage 3 were employed for all subsequent experiments.

Synthesis, characterization and sterilization of $PGMA_{63}$-$PHPMA_{165}$ worm gels

Materials. Glycerol monomethacrylate (GMA; 99.8%) was provided by GEO Specialty Chemicals (Hythe, UK) and used as received. 2-Hydroxypropyl methacrylate (HPMA) and 4,4'-azobis(4-cyanopentanoic acid) (ACVA, a.k.a. V-501; 99%) were obtained from Alfa Aesar (Heysham, UK). 2-Cyano-2-propyl dithiobenzoate (CPDB, 80% purity) was purchased from Strem Chemicals (Newton, UK). CD_3OD (99.8%) was sourced from Goss Scientific (Nantwich, UK). HPLC-grade solvents were purchased from Fisher Scientific (Loughborough, UK) and were used as received.

Synthesis of $PGMA_{63}$ precursor via RAFT solution polymerization of GMA in ethanol. Glycerol monomethacrylate (GMA, 208.2 g, 1.30 mol) and CPDB (4.43 g, 0.020 mol; target DP = 65) were weighed into a 1 L round-bottomed flask and purged using a stream of N_2 gas

for 20 min at 20 °C. Then ACVA (1.12 g, 4.0 mmol) was added and the resulting solution was degassed with N_2 gas for a further 5 min. Anhydrous ethanol (175 g, 3.8 mol) was also degassed prior to addition, with the reaction solution being degassed for 5 min prior to immersion of the reaction flask in a 70 °C oil bath. A proton NMR spectrum recorded in CD_3OD after 2 h indicated a GMA conversion of 80%. The crude polymer was isolated via precipitation into excess dichloromethane from methanol (twice) to remove traces of residual reactants. Filtration afforded the purified insoluble polymer, which was then dissolved in deionized water (200 ml). Traces of dichloromethane was removed by rotary evaporation at 30 °C and the resulting aqueous polymer solution was lyophilized for 16 h to produce a pink powder. A proton NMR spectrum of this polymer dissolved in CD_3OD was recorded and comparison of the relevant aromatic and aliphatic signals enabled a mean degree of polymerization of 63 to be calculated by end-group analysis. DMF GPC studies using a refractive index detector combined with a series of near-monodisperse poly(methyl methacrylate) calibration standards indicated an M_n of 14 600 g mol^{-1} and an M_w/M_n of 1.12.

Synthesis of $PGMA_{63}$-$PHPMA_{165}$ diblock copolymer worms via RAFT aqueous dispersion polymerization of HPMA in 0.15 M PBS. The $PGMA_{63}$ precursor (14.0 g, 1.36 mmol) and HPMA monomer (32.30 g, 0.22 mol) were each weighed into a 500 mL round-bottom flask and the resulting solution was purged with a stream of N_2 gas for 20 min. Then ACVA initiator (0.10 g, 0.33 mmol) was added, followed by further degassing for 5 min. An aqueous solution of phosphate-buffered saline (PBS) (Dulbecco A, Oxoid, Basingstoke, 185 g, 150 mM, previously degassed using a N_2 stream for 30 min) was added, prior to further degassing for 5 min. Then the reaction flask was immersed in a 70 °C oil bath to initiate the HPMA polymerization. A proton NMR spectrum (CD_3OD) of the reaction solution indicated essentially full HPMA conversion after 5 h, as judged by comparing the integrated vinyl signals observed at 5.6 and 6.2 ppm. DMF GPC studies using a refractive index detector combined with a series of poly(methyl methacrylate) calibration standards) indicated an M_n of 47 400 g mol^{-1} (M_w/M_n = 1.17). The resulting 20% w/w $PGMA_{63}$-$PHPMA_{165}$ worm gel was dialyzed (molecular weight cut-off = 3 500) at 4 °C against deionized water for twelve days, with dialyzate being replaced twice daily. The purified diblock copolymer was then lyophilized to afford a fine pink powder, which could be readily redispersed (initially at 4 °C, with magnetic stirring for at least 20 min) in the desired aqueous medium to obtain an appropriate copolymer concentration.[53] The resulting low-viscosity fluid was filter-sterilized prior to use, as described below.

Sterilization protocol. $PGMA_{63}$-$PHPMA_{165}$ worm gels (either 6% w/v or 10% w/v) prepared using the desired cell culture medium were cooled from 20 °C to 4 °C using an ice bath to induce the worm-to-sphere transition. The ensuing degelation produced a free-flowing aqueous dispersion of spherical copolymer nanoparticles in each case. Within a laminar flow cabinet, the resulting low-viscosity fluids were ultrafiltered into sterile vials using a syringe filter (0.22 μm). Both ultrafilters and syringes were stored for 60 min at −20 °C prior to

ultrafiltration to ensure that gelation did not occur on contact. On allowing these sterile copolymer aqueous dispersions to return to 20 °C, regelation was observed owing to worm reconstitution. The resulting worm gels were utilized immediately for ASC encapsulation experiments.

Rheology measurements. All experiments were conducted using a stress-controlled AR-G2 rheometer comprising a variable temperature Peltier plate and a 40 mm 2° aluminium cone. Storage and loss moduli were determined as a function of temperature, angular frequency and applied strain to assess gel strength and identify the critical gelation temperature (CGT). Thermal cycles (from 2 °C to 40 °C to 2 °C) were conducted at 1.0 rad s^{-1} using an applied strain of 1.0% with data being recorded at 2 °C intervals and 5 min being allowed for thermal equilibration at each temperature.

Encapsulation of ASCs in $PGMA_{63}$-$PHPMA_{165}$ worm gels

ASCs were suspended within worm gels (either 6% w/v or 10% w/v) at a density of 1×10^6 cells per ml. A series of 100 µL worm gels were obtained in 0.40 µm high-density cell culture inserts (BD Biosciences, San Jose, CA, USA). These cell-loaded gels were then cultured in standard media for 24 h. Subsequently, the initial media were replaced with an appropriate differentiating medium (see below for further details) in the presence or absence of a suitable growth factor.

Encapsulation of ASCs in type I collagen hydrogels

Collagen gels were prepared by mixing 0.465 ml type I collagen solution (6.45 µg gm^{-3}) (Devro, Edinburgh, Scotland) with neutralization buffer (0.10 mL 10x PBS, 10 µL 1 M NaOH, and 0.415 mL sterilized water). ASCs were dispersed within the collagen solution at 20 °C to produce a cell density of 1×10^6 per mL and 100 µL gels were formed in 0.40 µm high-density cell culture inserts. These gels were cultured using standard media for 24 h. Subsequently, the initial media were replaced with an appropriate differentiating medium (see below for further details) in the presence or absence of a suitable growth factor.

Differentiation of ASCs with TGF-β3 and GDF6

ASCs were encapsulated within type I collagen gels and then cultured using a differentiating medium comprising glucose-rich DMEM, 1.0% FCS, insulin-transferrin-selenium (ITS-X) (Gibco, Grand Island, NY, USA), 100 µM ascorbic acid-2-phosphate, 1.25 g dm^{-3} bovine serum albumin (BSA), 10^{-7} M dexamethasone, 5.4 ng dm^{-3} linoleic acid, 40 ng dm^{-3} L-proline, 100 U per mL penicillin, 100 ng dm^{-3} streptomycin, and 2.5 ng dm^{-3} amphotericin B. This medium was supplemented with either no growth factor (control), TGF-β3 (Invitrogen) at 10 pg dm^{-3}, or GDF6 (PeproTech) at 100 pg dm^{-3}. Media were replaced every 48 h for 14 days.

Metabolic activity of ASCs within collagen gels and worm gels

The metabolic activity of ASCs suspended within collagen gel, 6% w/v worm gel and 10% w/v worm gel, cultured in 21% O_2 at a density of 1 x 10^6 cells per mL was assessed using an Alamar Blue assay (Life Technologies, Paisley UK) in differentiation media after 1, 7 and 14 days according to the manufacturer's protocol. The fluorescence emission was recorded using a fluorescence microplate reader (TECAN) using excitation and emission wavelengths of 590 nm and 540 nm, respectively. Fluorescence was recorded for cellular constructs and normalized to that of acellular control constructs to provide an indication of either cytotoxicity or proliferation.

Assessment of NP marker gene expression with quantitative real-time PCR

After 14 days, cell-seeded collagen hydrogels and pellets were disrupted with Molecular Grinding Resin in TRIzol (Geno Technology Inc., St. Louis, MO, USA), and RNA was extracted according to the manufacturer's recommendations. cDNA was generated by using a high-capacity cDNA reverse-transcription kit (Life Technologies) and diluted to 5 ng/µl. Gene expression was analyzed by quantitative real-time PCR (qRT-PCR) using an Applied Biosystems StepOne Plus Real Time PCR system (Applied Biosystems) in order to investigate gene expression levels of NP markers; ACAN, COL2A, SOX9, cytokeratin-8 (KRT8), KRT-19 and KRT-19.

Experiments were conducted in triplicate by using LuminoCt qPCR readymix reagents (Sigma-Aldrich, Irvine, UK) to a total volume of 10 µl, containing 10 ng cDNA, 900 nM each primer, and 250 nM probe. Data were analyzed according to the 2-ΔCt method, with expression normalized to the average of two prevalidated housekeeping genes (EIF2B1and GAPDH) and to the respective sample control (unstimulated).

Statistical analysis

Data were assessed for normality using the Shapiro Wilks test and found to be non-parametric. Hence statistical comparisons were performed by Kruskal-Wallis with a pairwise comparisons (Conover-Inman) post hoc test performed with statistical significance accepted at $p < 0.05$.

Results and Discussion

Synthesis and aqueous solution behavior of the $PGMA_{63}$-$PHPMA_{165}$ worms

The chemical structure of the $PGMA_{63}$-$PHPMA_{165}$ diblock copolymer and its corresponding $PGMA_{63}$ homopolymer precursor is shown in Figure 1a. DMF GPC analysis indicated a high blocking efficiency was achieved during the RAFT aqueous dispersion polymerization of HPMA, with minimal contamination by the $PGMA_{63}$ homopolymer precursor. On drying 0.2% w/w aqueous dispersions of $PGMA_{63}$-$PHPMA_{165}$ nano-objects (prepared in the presence of the αMEM cell culture medium used in this study), TEM analysis indicated a pseudo-spherical morphology at 4 °C, whereas highly anisotropic worms are present at 21 °C (see

Figure 1b). These observations are consistent with those reported by Blanazs et al.[49]The latter nanoparticles form a relatively soft 3D gel network owing to multiple inter-worm contacts[52]. The temperature dependence of the gel modulus (G' and G'' are denoted by red and blue squares, respectively) is shown in Figure 1c for a 10% w/w aqueous dispersion of $PGMA_{63}$-$PHPMA_{165}$ worms. These data confirm that degelation occurs on cooling the worm gel and regelation occurs on heating, albeit with some hysteresis. Similar observations have been reported by Verber et al. for $PGMA_{54}$-$PHPMA_{150}$ worms [51]Such thermoreversibility is critical for potential biomedical applications because it enables facile sterilization via cold ultrafiltration.[49]

Metabolic activity of ASCs

The Alamar Blue assay is a measure of metabolic cell activity and thus the total number of viable cells was assessed over 14 days in culture (Fig. 2). The ASCs remained metabolically active when encapsulated within 6% or 10% w/v worm gels and cultured in 21% O_2, with significant increases in activity being observed at day 7 and 14 compared to day 1 (Fig. 2).

NP marker expression in type I collagen hydrogels

Culture of ASCs in the presence of growth factors (TGFß or GDF6) resulted in a significant upregulation of ACAN and KRT18, with non-significant upregulations in COL2A, SOX-9 and KRT19 (Fig. 3).

NP marker expression within the $PGMA_{63}$-$PHPMA_{165}$ worm gel

Culture of ASCs encapsulated in 6% w/v worm gels demonstrated significant upregulations in ACAN (Figure 3) when cultured in the presence of either GDF6 or TGFß compared to the absence of any growth factor. COL2A expression was significantly higher in cells cultured in the presence of TGFß compared to GDF6, and also compared to unstimulated cells (Figure 3). Levels of SOX-9, KRT8 and KRT18 remained stable, regardless of the addition of growth factors. However, KRT19 mRNA expression was significantly reduced by stimulation with GDF6 in 6% worm gels (Figure 3). It is noteworthy that gene levels of SOX9 and KRT18 were higher in cells encapsulated within 6% w/v worm gels than those within collagen gels, whether in the presence or absence of growth factors.

Similarly, ASCs encapsulated in 10% w/v worm gels expressed significantly higher levels of ACAN (Figure 3) when cultured in the presence of GDF6, whereas significant down regulations were evident for expression levels of KRT8 and KRT18 in cells stimulated with TGFß (Figure 3). However, expression levels of ACAN, COL2A, SOX9 and KRT8 and KRT18 (Figure 3A-F) were always higher in cells encapsulated and cultured in the 10% w/v worm gel compared to the collagen and 6% w/v worm gel. This was evident even for cells that were not cultured in the presence of growth factors.

The use of MSCs for regeneration of the IVD is currently under investigation, with pre-clinical and phase I clinical trials indicating the safety and efficacy of allogeneic [56] and

autologous [57-60] MSCs injected into the degenerate IVD. Current therapies for IVD degeneration include both conservative and surgical treatments. However, such therapies have a variable success rate, with many patients experiencing accelerated adjacent-disk degeneration within two years of surgery[61].

To provide a safe, successful therapy for patients with IVD degeneration, an optimized protocol is required to deliver these cells to the site of degeneration, with such cells then repopulating the NP as well as generating new ECM to re-establish tissue integrity. Here we demonstrate that relatively soft physical hydrogels are a potential carrier for MSCs, which can influence differentiation of human ASCs towards an NP phenotype. The chemical structure of the PGMA-PHPMA worm gels used in this study bear a striking resemblance to the GAGs that are abundant within the IVD ECM. More specifically, such worm gels exhibit bulk moduli (G') of approximately 18-86 Pa in PBS buffer for copolymer concentrations ranging from 6% to 10% w/w, respectively. It is well-documented that matrix stiffness influences the focal-adhesion structure and the cytoskeleton of MSCs[62, 63]. However, pioneering studies conducted by Engler *et al.* demonstrated the importance of substrate stiffness in directing stem cell differentiation down various lineages. Thus, cells grown on softer substrates that are characteristic of brain tissue (0.1-1 kPa) up-regulated factors involved in neurogenesis, while cells grown on stiffer matrices (25-40 kPa) that mimic the cross-linked collagen of osteoids yielded MSCs with the characteristic morphology of osteoblasts[63].

The elastic shear modulus of the human NP has been widely studied and is reported to be in the range of 0.1 – 1 kPa[64-67]. During the degenerative process, the NP becomes progressively more cross-linked during the transition from type II collagen to a more fibrous type I collagen. Thus the NP should become stiffer, which should modulate the cellular response. Many studies investigating MSC differentiation capacity toward an NP-phenotype for regeneration have aimed to recapitulate the ECM stiffness of the naïve IVD, which is primarily composed of ACAN and COL2A. Navaro *et al.* examined the effect of matrix stiffness on NP-derived stem cells by comparing matrices with G' values of 1 kPa and 2 kPa. It was found that weaker hydrogels (Tetronic-fibrinogen) exhibiting moduli comparable to the physiological modulus found in native IVD promoted cell survival and chondrogenic differentiation, as well as enhanced cell attachment to the matrix[68]. Thorpe *et al.* reported site-directed differentiation of human BMSCs toward an NP phenotype when incorporated into a pNIPAM-DMAc-Laponite® hydrogel with a G' of 5.5 x 10^5 Pa under compression mode[69]. Although this hydrogel has a stiffness comparable to that of bone, the encapsulation of human BMSCs under hypoxic (5% O_2) conditions for up to six weeks demonstrated remarkable protein expression profiles that were characteristic of an NP phenotype. This literature precedent demonstrates the importance of mimicking the physiological conditions of the IVD when performing such differentiation experiments. Studies conducted on softer gels such as commercially available *Matrigel*™ confirmed the transition of umbilical cord stem cells to an immature NP phenotype[70], as indicated by

greater expression of immature NP cells markers during differentiation, as well as ECM proteins, GAGs and laminin receptor proteins. *Matrigel*™ typically exhibits a G' value of approximately 120-450 Pa, which is appreciably higher compared to the G' of the worm gels used in the present study. Thus, further work is warranted using worm gels with bulk moduli closer to that of *Matrigel*™ to examine whether a similar immature NP phenotype could be obtained.

The current study demonstrates that an NP-like mRNA expression profile can be achieved within significantly softer materials than those previously reported[8, 69, 71-73]. ASCs exhibit an increase in NP marker genes (ACAN and COL2A) when cultured in worm gels for 14 days *even in the absence of any growth factors*. Growth factors belonging to the TGFß superfamily have been used in many studies to promote differentiation via the chondrogenic lineage. This is particularly true for TGFß3, which is known for its anabolic effects on matrix synthesis[74]. In prior studies, Clarke *et al.* reported the preferential use of GDF6 for stimulating differentiation of ASCs encapsulated within collagen gels into NP cells after 14 days of cell culture[45]. Despite the addition of growth factors (TGFß3 or GDF6), we demonstrate that NP marker expression levels are higher in ASCs encapsulated and cultured within worm gels compared to the same cells within collagen gels. Thus, ASCs enhance their expression of phenotypic markers in response to the mechanically softer, hydroxyl-rich environment offered by the worm gel, which is more representative of the native NP environment. Further work is warranted to examine whether protein expression of NP markers is also upregulated in ASCs encapsulated within these worm gels. One reviewer of this manuscript suggested that the presence of the synthetic hydrogel might affect the quality of the regenerated NP and perhaps also change the mechanical properties of the final tissue. This is because healthy NP tissue comprises 65 % proteoglycan and 20% collagen, hence no more than approximately 15% can be water. Thus, if the injected hydrogel already contributes 10% of the allowable aqueous volume this might be problematic for the regenerated tissue. However, we seek to develop an injectable hydrogel to regenerate damaged NPs. Such compromised NPs are known to contain pores/fissures owing to loss/degradation of tissue during their degeneration[75]. Accordingly, there should be *additional* volume available for a synthetic hydrogel to occupy compared to that for a healthy NP.

CONCLUSIONS

In summary, adipose-derived mesenchymal stem cells (ASCs) can be readily encapsulated within hydroxyl-functional thermoresponsive diblock copolymer worm gels, which form relatively soft biocompatible gels above 21°C. Moreover, these shear-thinning worm gels can be injected into the IVD using a 21G needle, which is a fundamental requirement for any potential cell delivery system. Furthermore, degelation can be induced on demand simply by cooling, which allows (i) facile sterilization via cold ultrafiltration and (ii) convenient

harvesting of the cells during *in vitro* experiments. In cell culture studies conducted using such wholly synthetic worm gels, human ASCs express higher levels of NP markers, particularly aggrecan, relative to control experiments conducted using collagen gels. Since they resemble key components of the native NP matrix, these biocompatible worm gels offer considerable potential as an injectable cell delivery system and scaffold for the potential treatment of degenerate disk disease.

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ASSOCIATED CONTENT

Supporting Information Available. Digital photographs illustrating the shear-thinning injectable nature of the block copolymer worm gel used in this study. This material is available free of charge via the Internet at http://pubs.acs.org.

References

1. Shoichet, M. S. Polymer scaffolds for biomaterials applications. *Macromolecules* **2010**, *43,* 581-591.

2. Nimmo, C. M.; Owen, S. C.; Shoichet, M. S. Diels−Alder click cross-linked hyaluronic acid hydrogels for tissue engineering. *Biomacromolecules* **2011**, *12,* 824-830.

3. Gupta, D.; Tator, C. H.; Shoichet, M. S. Fast-gelling injectable blend of hyaluronan and methylcellulose for intrathecal, localized delivery to the injured spinal cord. *Biomaterials* **2006**, *27,* 2370-2379.

4. Li, T.; Huang, F.; Diaz-Dussan, D.; Zhao, J.; Srinivas, S.; Narain, R.; Tian, W.; Hao, X. Preparation and characterization of thermoresponsive PEG-based injectable hydrogels and their application for 3D cell culture. *Biomacromolecules* **2020**, *21,* 1254-1263.

5. Chen, Y.; Tan, Z.; Wang, W.; Peng, Y.; Narain, R. Injectable, self-healing, and multi-responsive hydrogels via dynamic covalent bond formation between benzoxaborole and hydroxyl groups. *Biomacromolecules* **2018**, *20,* 1028-1035.

6. Yoshikawa, H. Y.; Rossetti, F. F.; Kaufmann, S.; Kaindl, T.; Madsen, J.; Engel, U.; Lewis, A. L.; Armes, S. P.; Tanaka, M. Quantitative evaluation of mechanosensing of cells on dynamically tunable hydrogels. *J. Am. Chem. Soc.* **2011**, *133,* 1367-1374.

7. O'Halloran, D. M.; Pandit, A. S. Tissue-engineering approach to regenerating the intervertebral disc. *Tissue Eng.* **2007**, *13,* 1927-1954.

8. Calderon, L.; Collin, E.; Murphy, M.; O'Halloran, D.; Pandit, A. Type II collagen-hyaluronan hydrogel-a step towards a scaffold for intervertebral disc tissue engineering. *Eur. Cells Mater.* **2010**, *20*, 134-148

9. Vickers, L.; Thorpe, A. A.; Snuggs, J.; Sammon, C.; Le Maitre, C. L. Mesenchymal stem cell therapies for intervertebral disc degeneration: Consideration of the degenerate niche. *JOR Spine* **2019**, *2,* e1055.

10. Thorpe, A.; Dougill, G.; Vickers, L.; Reeves, N.; Sammon, C.; Cooper, G.; Le Maitre, C. Thermally triggered hydrogel injection into bovine intervertebral disc tissue explants induces differentiation of mesenchymal stem cells and restores mechanical function. *Acta Biomater.* **2017**, *54,* 212-226.

11. Collin, E. C.; Grad, S.; Zeugolis, D. I.; Vinatier, C. S.; Clouet, J. R.; Guicheux, J. J.; Weiss, P.; Alini, M.; Pandit, A. S. An injectable vehicle for nucleus pulposus cell-based therapy. *Biomaterials* **2011**, *32,* 2862-2870.

12. Halloran, D. O.; Grad, S.; Stoddart, M.; Dockery, P.; Alini, M.; Pandit, A. S. An injectable cross-linked scaffold for nucleus pulposus regeneration. *Biomaterials* **2008**, *29,* 438-447.

13. Isa, I. L. M.; Srivastava, A.; Tiernan, D.; Owens, P.; Rooney, P.; Dockery, P.; Pandit, A. Hyaluronic acid based hydrogels attenuate inflammatory receptors and neurotrophins in interleukin-1β induced inflammation model of nucleus pulposus cells. *Biomacromolecules* **2015**, *16,* 1714-1725.

14. Li, Z.; Lang, G.; Karfeld-Sulzer, L. S.; Mader, K. T.; Richards, R. G.; Weber, F. E.; Sammon, C.; Sacks, H.; Yayon, A.; Alini, M. Heterodimeric BMP-2/7 for nucleus pulposus regeneration—In vitro and ex vivo studies. *J. Orthopaedic Res.* **2017**, *35,* 51-60.

15. Pérez-San Vicente, A.; Peroglio, M.; Ernst, M.; Casuso, P.; Loinaz, I.; Grande, H.; Alini, M.; Eglin, D.; Dupin, D. Self-healing dynamic hydrogel as injectable shock-absorbing artificial nucleus pulposus. *Biomacromolecules* **2017**, *18,* 2360-2370.

16. Gan, Y.; Li, P.; Wang, L.; Mo, X.; Song, L.; Xu, Y.; Zhao, C.; Ouyang, B.; Tu, B.; Luo, L. An interpenetrating network-strengthened and toughened hydrogel that supports cell-based nucleus pulposus regeneration. *Biomaterials* **2017**, *136,* 12-28.

17. Allen, M. J.; Schoonmaker, J. E.; Bauer, T. W.; Williams, P. F.; Higham, P. A.; Yuan, H. A. Preclinical evaluation of a poly(vinyl alcohol) hydrogel implant as a replacement for the nucleus pulposus. *Spine* **2004**, *29,* 515-523.

18. Chou, A. I.; Akintoye, S. O.; Nicoll, S. B. Photo-crosslinked alginate hydrogels support enhanced matrix accumulation by nucleus pulposus cells in vivo. *Osteoarthritis Cartilage* **2009**, *17,* 1377-1384.

19. Reza, A. T.; Nicoll, S. B. Characterization of novel photocrosslinked carboxymethylcellulose hydrogels for encapsulation of nucleus pulposus cells. *Acta Biomater.* **2010**, *6,* 179-186.

20. Su, W.; Chen, Y.; Lin, F. Injectable oxidized hyaluronic acid/adipic acid dihydrazide hydrogel for nucleus pulposus regeneration. *Acta Biomater.* **2010**, *6,* 3044-3055.

21. Chen, P.; Ning, L.; Qiu, P.; Mo, J.; Mei, S.; Xia, C.; Zhang, J.; Lin, X.; Fan, S. Photo-crosslinked gelatin-hyaluronic acid methacrylate hydrogel-committed nucleus pulposus-like differentiation of adipose stromal cells for intervertebral disc repair. *J. Tissue Eng. Regenerative Med.* **2019**, *13,* 682-693.

22. Wang, F.; Nan, L.; Zhou, S.; Liu, Y.; Wang, Z.; Wang, J.; Feng, X.; Zhang, L. Injectable hydrogel combined with nucleus pulposus-derived mesenchymal stem cells for the treatment of degenerative intervertebral disc in rats. *Stem Cells Int.,* **2019**, 8496025.

23. Cloyd, J. M.; Malhotra, N. R.; Weng, L.; Chen, W.; Mauck, R. L.; Elliott, D. M. Material properties in unconfined compression of human nucleus pulposus, injectable hyaluronic acid-based hydrogels and tissue engineering scaffolds. *Eur. Spine Journal* **2007**, *16,* 1892-1898.

24. Maniadakis, N.; Gray, A. The economic burden of back pain in the UK. *Pain* **2000**, *84,* 95-103.

25. Juniper, M.; Le, T. K.; Mladsi, D. The epidemiology, economic burden, and pharmacological treatment of chronic low back pain in france, germany, italy, spain and the UK: A literature-based review. *Expert Opin. Pharmacother.* **2009**, *10,* 2581-2592.

26. Hutton, W. C.; Ganey, T. M.; Elmer, W. A.; Kozlowska, E.; Ugbo, J. L.; Doh, E. S.; Whitesides, T. E.,Jr. Does long-term compressive loading on the intervertebral disc cause degeneration? *Spine* **2000**, *25,* 2993-3004.

27. Cherkin, D. C.; Wheeler, K. J.; Barlow, W.; Deyo, R. A. Medication use for low back pain in primary care. *Spine* **1998**, *23,* 607-614.

28. Malanga, G. A.; Nadler, S. F. Nonoperative treatment of low back pain. *Mayo Clin. Proc.* **1999**, *74,* 1135-1148.

29. Cherkin, D. C.; Deyo, R. A.; Battie, M.; Street, J.; Barlow, W. A comparison of physical therapy, chiropractic manipulation, and provision of an educational booklet for the treatment of patients with low back pain. *N. Engl. J. Med.* **1998,** *339,* 1021-1029.

30. Mirza, S. K.; Deyo, R. A. Systematic review of randomized trials comparing lumbar fusion surgery to nonoperative care for treatment of chronic back pain. *Spine* **2007,** *32,* 816-823.

31. Chou, R.; Huffman, L. H. Medications for acute and chronic low back pain: A review of the evidence for an american pain society/american college of physicians clinical practice guideline. *Ann. Intern. Med.* **2007,** *147,* 505-514.

32. Chou, R.; Loeser, J. D.; Owens, D. K.; Rosenquist, R. W.; Atlas, S. J.; Baisden, J.; Carragee, E. J.; Grabois, M.; Murphy, D. R.; Resnick, D. K.; Stanos, S. P.; Shaffer, W. O.; Wall, E. M.; American Pain Society Low Back Pain Guideline Panel. Interventional therapies, surgery, and interdisciplinary rehabilitation for low back pain: An evidence-based clinical practice guideline from the american pain society. *Spine* **2009,** *34,* 1066-1077.

33. Vedicherla, S.; Buckley, C. T. Cell-based therapies for intervertebral disc and cartilage regeneration—Current concepts, parallels, and perspectives. *J. Orthop. Res.* **2017**, *35,* 8-22.

34. Iatridis, J. C.; Kang, J.; Kandel, R.; Risbud, M. V. New horizons in spine research: Intervertebral disc repair and regeneration. *J. Orthop. Res.* **2017**, *35,* 5-7.

35. Buckley, C. T.; Hoyland, J. A.; Fujii, K.; Pandit, A.; Iatridis, J. C.; Grad, S. Critical aspects and challenges for intervertebral disc repair and regeneration—Harnessing advances in tissue engineering. *JOR Spine* **2018**, *1,* e1029.

36. Lee, C. R.; Sakai, D.; Nakai, T.; Toyama, K.; Mochida, J.; Alini, M.; Grad, S. A phenotypic comparison of intervertebral disc and articular cartilage cells in the rat. *Eur. Spine J.* **2007**, *16,* 2174-2185.

37. Chelberg, M. K.; Banks, G. M.; Geiger, D. F.; Oegema, T. R., Jr. Identification of heterogeneous cell populations in normal human intervertebral disc. *J. Anat.* **1995**, *186 (Pt 1),* 43-53.

38. Mwale, F.; Roughley, P.; Antoniou, J. Distinction between the extracellular matrix of the nucleus pulposus and hyaline cartilage: A requisite for tissue engineering of intervertebral disc. *Eur. Cell. Mater.* **2004**, *8,* 58-63; discussion 63-4.

39. Minogue, B. M.; Richardson, S. M.; Zeef, L. A.; Freemont, A. J.; Hoyland, J. A. Characterization of the human nucleus pulposus cell phenotype and evaluation of novel marker gene expression to define adult stem cell differentiation. *Arthritis Rheum.* **2010**, *62,* 3695-3705.

40. Rodrigues-Pinto, R.; Richardson, S. M.; Hoyland, J. A. Identification of novel nucleus pulposus markers: Interspecies variations and implications for cell-based therapiesfor intervertebral disc degeneration. *Bone Joint Res.* **2013**, *2,* 169-178.

41. Risbud, M. V.; Schoepflin, Z. R.; Mwale, F.; Kandel, R. A.; Grad, S.; Iatridis, J. C.; Sakai, D.; Hoyland, J. A. Defining the phenotype of young healthy nucleus pulposus cells: Recommendations of the spine research interest group at the 2014 annual ORS meeting. *J. Orthop. Res.* **2015**, *33,* 283-293.

42. Thorpe, A. A.; Binch, A. L.; Creemers, L. B.; Sammon, C.; Le Maitre, C. L. Nucleus pulposus phenotypic markers to determine stem cell differentiation: Fact or fiction? *Oncotarget* **2016**, *7,* 2189-2200.

43. Le Maitre, C. L.; Freemont, A. J.; Hoyland, J. A. Expression of cartilage-derived morphogenetic protein in human intervertebral discs and its effect on matrix synthesis in degenerate human nucleus pulposus cells. *Arthritis Res. Ther.* **2009**, *11,* R137.

44. Hodgkinson, T.; Shen, B.; Diwan, A.; Hoyland, J. A.; Richardson, S. M. Therapeutic potential of growth differentiation factors in the treatment of degenerative disc diseases. *JOR Spine* **2019**, e1045.

45. Clarke, L. E.; McConnell, J. C.; Sherratt, M. J.; Derby, B.; Richardson, S. M.; Hoyland, J. A. Growth differentiation factor 6 and transforming growth factor-beta differentially mediate mesenchymal stem cell differentiation, composition, and micromechanical properties of nucleus pulposus constructs. *Arthritis Res. Ther.* **2014**, *16,* R67.

46. Thorpe, A. A.; Bach, F. C.; Tryfonidou, M. A.; Le Maitre, C. L.; Mwale, F.; Diwan, A. D.; Ito, K. Leaping the hurdles in developing regenerative treatments for the intervertebral disc from preclinical to clinical. *JOR Spine* **2018**, *1,* e1027.

47. Sakai, D.; Schol, J. Cell therapy for intervertebral disc repair: Clinical perspective. *J. Orthopaedic Transl.* **2017**, *9,* 8-18.

48. Sakai, D.; Grad, S. Advancing the cellular and molecular therapy for intervertebral disc disease. *Adv. Drug Deliv. Rev.* **2015**, *84,* 159-171.

49. Blanazs, A.; Verber, R.; Mykhaylyk, O. O.; Ryan, A. J.; Heath, J. Z.; Douglas, C. I.; Armes, S. P. Sterilizable gels from thermoresponsive block copolymer worms. *J. Am. Chem. Soc.* **2012**, *134,* 9741-9748.

50. Ratcliffe, L. P.; Couchon, C.; Armes, S. P.; Paulusse, J. M. Inducing an order–order morphological transition via chemical degradation of amphiphilic diblock copolymer nano-objects. *Biomacromolecules* **2016**, *17,* 2277-2283.

51. Verber, R.; Blanazs, A.; Armes, S. Rheological studies of thermo-responsive diblock copolymer worm gels. *Soft Matter* **2012**, *8,* 9915-9922.

52. Lovett, J. R.; Derry, M. J.; Yang, P.; Hatton, F. L.; Warren, N. J.; Fowler, P. W.; Armes, S. P. Can percolation theory explain the gelation behavior of diblock copolymer worms? *Chem. Sci.* **2018**, *9,* 7138-7144.

53. Canton, I.; Warren, N. J.; Chahal, A.; Amps, K.; Wood, A.; Weightman, R.; Wang, E.; Moore, H.; Armes, S. P. Mucin-inspired thermoresponsive synthetic hydrogels induce stasis in human pluripotent stem cells and human embryos. *ACS Cent. Sci.* **2016**, *2,* 65-74.

54. Kocik, M.; Mykhaylyk, O.; Armes, S. Aqueous worm gels can be reconstituted from freeze-dried diblock copolymer powder. *Soft Matter* **2014,** *10,* 3984-3992.

55. Burrow, K. L.; Hoyland, J. A.; Richardson, S. M. Human adipose-derived stem cells exhibit enhanced proliferative capacity and retain multipotency longer than donor-matched bone marrow mesenchymal stem cells during expansion in vitro. *Stem Cells Int.* **2017**, 2541275

56. Noriega, D. C.; Ardura, F.; Hernandez-Ramajo, R.; Martin-Ferrero, M. A.; Sanchez-Lite, I.; Toribio, B.; Alberca, M.; Garcia, V.; Moraleda, J. M.; Sanchez, A.; Garcia-Sancho, J. Intervertebral disc repair by allogeneic mesenchymal bone marrow cells: A randomized controlled trial. *Transplantation* **2017**, *101,* 1945-1951.

57. Yoshikawa, T.; Ueda, Y.; Miyazaki, K.; Koizumi, M.; Takakura, Y. Disc regeneration therapy using marrow mesenchymal cell transplantation: A report of two case studies. *Spine* **2010**, *35,* E475-E480.

58. Orozco, L.; Soler, R.; Morera, C.; Alberca, M.; Sanchez, A.; Garcia-Sancho, J. Intervertebral disc repair by autologous mesenchymal bone marrow cells: A pilot study. *Transplantation* **2011**, *92,* 822-828.

59. Elabd, C.; Centeno, C. J.; Schultz, J. R.; Lutz, G.; Ichim, T.; Silva, F. J. Intra-discal injection of autologous, hypoxic cultured bone marrow-derived mesenchymal stem cells in five patients with chronic lower back pain: A long-term safety and feasibility study. *J. Transl. Medicine* **2016**, *14,* 253.

60. Centeno, C.; Markle, J.; Dodson, E.; Stemper, I.; Williams, C. J.; Hyzy, M.; Ichim, T.; Freeman, M. Treatment of lumbar degenerative disc disease-associated radicular pain with culture-expanded autologous mesenchymal stem cells: A pilot study on safety and efficacy. *J. Transl. Medicine* **2017**, *15,* 197.

61. Hilibrand, A. S.; Robbins, M. Adjacent segment degeneration and adjacent segment disease: The consequences of spinal fusion? *The Spine J.* **2004**, *4,* S190-S194.

62. Discher, D. E.; Janmey, P.; Wang, Y. L. Tissue cells feel and respond to the stiffness of their substrate. *Science* **2005**, *310,* 1139-1143.

63. Engler, A. J.; Sen, S.; Sweeney, H. L.; Discher, D. E. Matrix elasticity directs stem cell lineage specification. *Cell* **2006**, *126,* 677-689.

64. Neidlinger-Wilke, C.; Galbusera, F.; Pratsinis, H.; Mavrogonatou, E.; Mietsch, A.; Kletsas, D.; Wilke, H. Mechanical loading of the intervertebral disc: From the macroscopic to the cellular level. *Eur. Spine J.* **2014**, *23,* 333-343.

65. Gilchrist, C. L.; Darling, E. M.; Chen, J.; Setton, L. A. Extracellular matrix ligand and stiffness modulate immature nucleus pulposus cell-cell interactions. *PLoS One* **2011**, *6,* e27170.

66. Setton, L. A.; Chen, J. Cell mechanics and mechanobiology in the intervertebral disc. *Spine* **2004**, *29,* 2710-2723.

67. Setton, L. A.; Chen, J. Mechanobiology of the intervertebral disc and relevance to disc degeneration. *J. Bone Joint Surg. Am.* **2006**, *88 Suppl 2,* 52-57.

68. Navaro, Y.; Bleich-Kimelman, N.; Hazanov, L.; Mironi-Harpaz, I.; Shachaf, Y.; Garty, S.; Smith, Y.; Pelled, G.; Gazit, D.; Seliktar, D. Matrix stiffness determines the fate of nucleus pulposus–derived stem cells. *Biomaterials* **2015**, *49,* 68-76.

69. Thorpe, A. A.; Boyes, V. L.; Sammon, C.; Le Maitre, C. L. Thermally triggered injectable hydrogel, which induces mesenchymal stem cell differentiation to nucleus pulposus cells: Potential for regeneration of the intervertebral disc. *Acta Biomater.* **2016**, *36*, 99-111.

70. Chon, B. H.; Lee, E. J.; Jing, L.; Setton, L. A.; Chen, J. Human umbilical cord mesenchymal stromal cells exhibit immature nucleus pulposus cell phenotype in a laminin-rich pseudo-three-dimensional culture system. *Stem Cell Res. Therapy* **2013**, *4,* 120.

71. Tsaryk, R.; Silva-Correia, J.; Oliveira, J. M.; Unger, R. E.; Landes, C.; Brochhausen, C.; Ghanaati, S.; Reis, R. L.; Kirkpatrick, C. J. Biological performance of cell-encapsulated methacrylated gellan gum-based hydrogels for nucleus pulposus regeneration. *J. Tissue Eng. Regen. Med.* **2017**, *11,* 637-648.

72. Richardson, S. M.; Hughes, N.; Hunt, J. A.; Freemont, A. J.; Hoyland, J. A. Human mesenchymal stem cell differentiation to NP-like cells in chitosan-glycerophosphate hydrogels. *Biomaterials* **2008**, *29,* 85-93.

73. Kumar, D.; Gerges, I.; Tamplenizza, M.; Lenardi, C.; Forsyth, N. R.; Liu, Y. Three-dimensional hypoxic culture of human mesenchymal stem cells encapsulated in a photocurable, biodegradable polymer hydrogel: A potential injectable cellular product for nucleus pulposus regeneration. *Acta Biomater.* **2014,** *10,* 3463-3474.

74. Barry, F.; Boynton, R. E.; Liu, B.; Murphy, J. M. Chondrogenic differentiation of mesenchymal stem cells from bone marrow: Differentiation-dependent gene expression of matrix components. *Exp. Cell Res.* **2001,** *268,* 189-200.

75. Le Maitre, C. L.; Freemont, A. J.; Hoyland, J. A. The role of interleukin-1 in the pathogenesis of human intervertebral disc degeneration. *Arthritis Res.Ther.* **2005,** *7,* R732-745.

Figures:

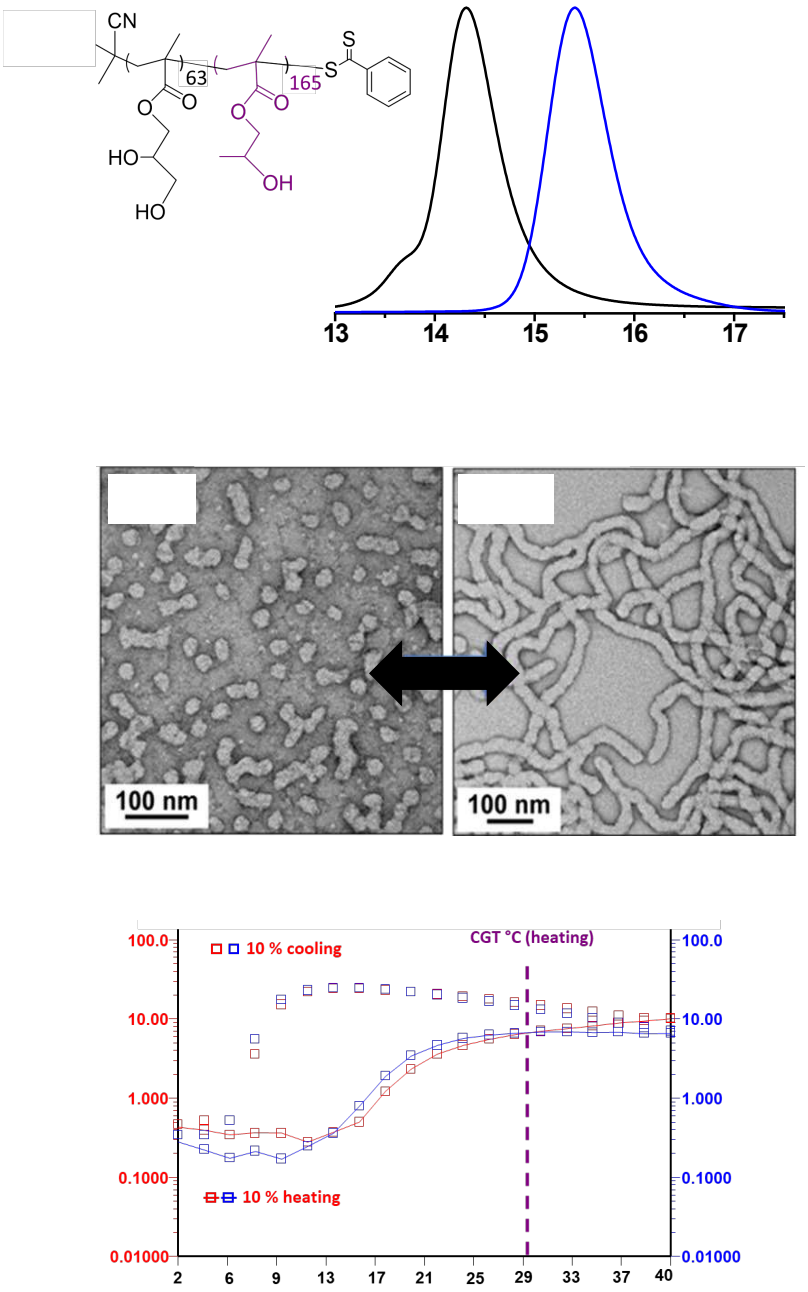

Figure 1. (a) Chemical structure and DMF GPC curves recorded for a $PGMA_{63}$-$PHPMA_{165}$ diblock copolymer and its corresponding $PGMA_{63}$ homopolymer precursor. (b) Representative TEM images recorded after drying a 0.2% w/w aqueous dispersion of $PGMA_{63}$-$PHPMA_{165}$ nano-objects (prepared in the presence of the αMEM cell culture medium used in this study). A pseudo-spherical morphology is observed at 4 °C while highly anisotropic worms are formed at 21 °C. The latter nanoparticles form a relatively soft 3D gel network owing to multiple inter-worm contacts. (c) Variation in gel modulus (G' and G'' are denoted by red and blue squares, respectively) with temperature for a 10% w/w aqueous dispersion of $PGMA_{63}$-$PHPMA_{165}$ worms at 1.0% applied strain and an angular frequency of 1.0 rad s^{-1}. Sweeps were conducted for a 2-40-2 °C thermal cycle with data acquired at 2 °C intervals and 5 min being allowed for equilibration between each measurement. Moduli recorded during the heating cycle are indicated by the solid red and blue lines and the purple dashed line corresponds to cross-over for the G' and G'' curves.

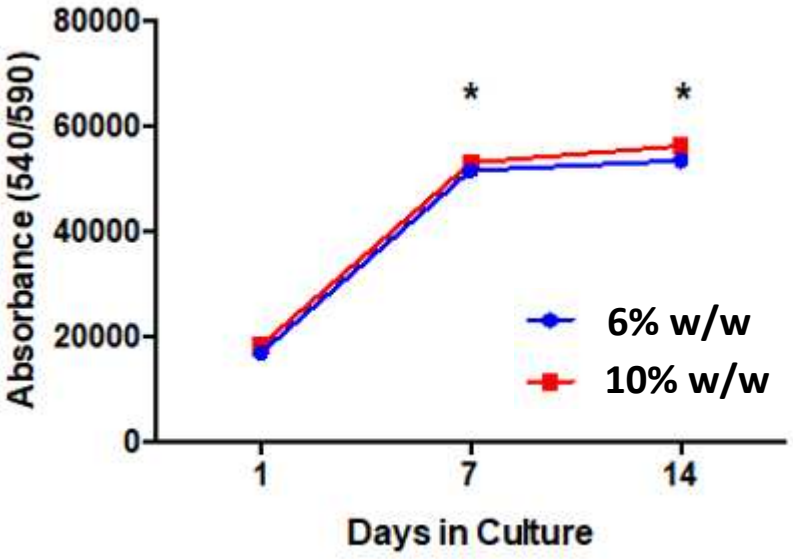

Figure 2. Metabolic activity of human ASCs (normalized to acellular controls) encapsulated within $PGMA_{63}$-$PHPMA_{165}$ worm gels at either 6% w/w or 10% w/w over a 14-day culture period. Statistical differences are indicated by * $P < 0.05$ compared to acellular controls.

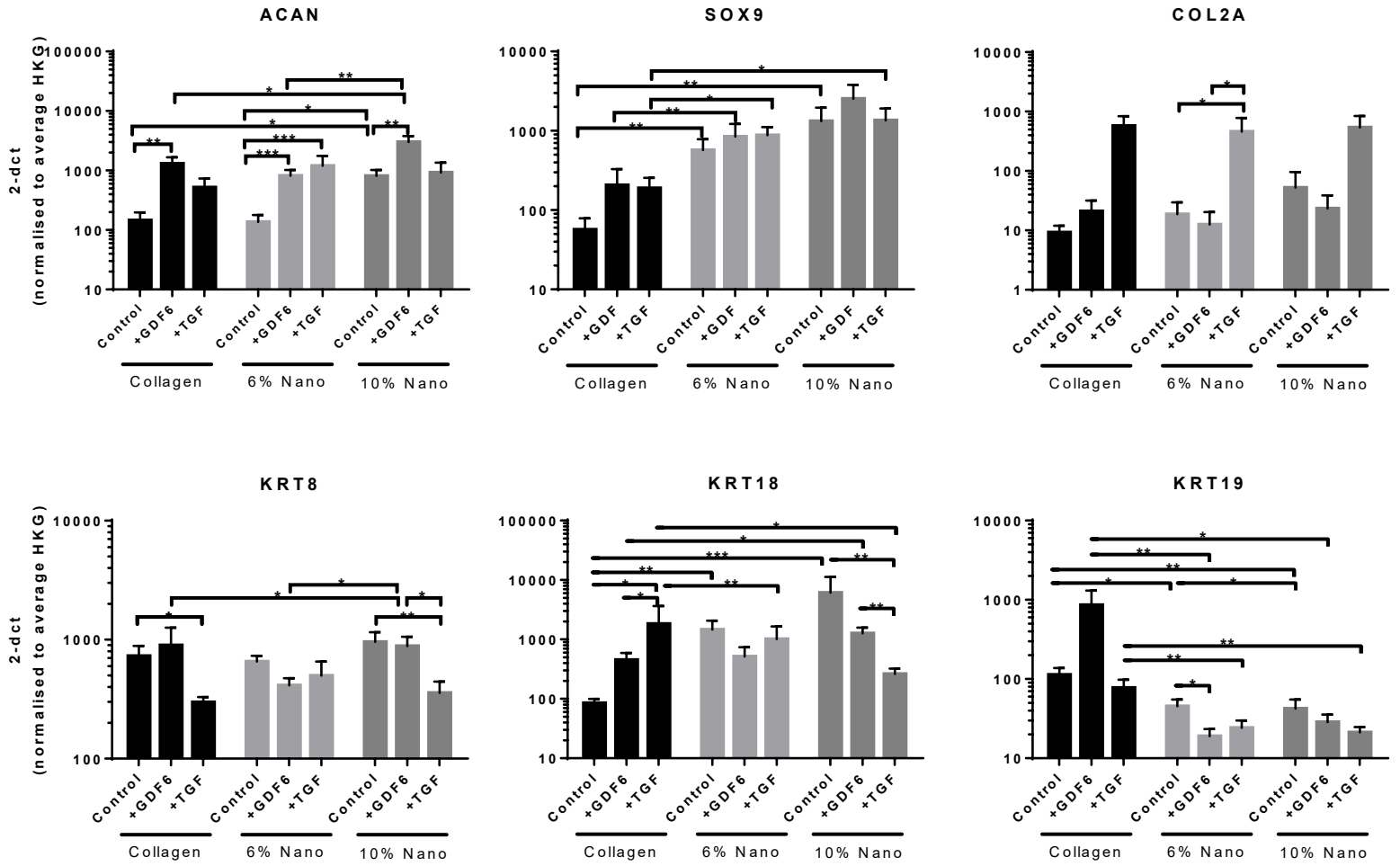

Figure 3. Quantitative real-time PCR analysis of NP marker gene expression from ASCs encapsulated in either collagen, 6% or 10% w/w worm gels ('Nano') over a 14-day culture period. Gene expression was normalized to that of reference genes (EIF2B1 and GAPDH). Values represent the mean ± SEM. Statistical significance determined using Kruskall-Wallis with Conover Inman multiple comparisons test, * = $P \leq 0.05$; ** = $P \leq 0.005$; *** = $P \leq 0.0005$ (n = 3).