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Mesenchymal Stem Cell Fate is Regulated by the Composition and Mechanical Properties of Collagen-Glycosaminoglycan Scaffolds

Ciara M. Murphy [1, 2], Amos Matsiko [1, 2], Matthew G. Haugh [1, 2], John P. Gleeson [1, 2], Fergal J. O'Brien [1, 2 *]

[1] Department of Anatomy, Royal College of Surgeons in Ireland, 123 St. Stephens Green, Dublin 2, Ireland

[2] Trinity Centre for Bioengineering, Trinity College Dublin, College Green, Dublin 2, Ireland

[*] Corresponding author: Present address: Department of Anatomy, Royal College of Surgeons in Ireland, 123 St. Stephens Green, Dublin 2, Ireland. Tel: +353(0)1-402-2149; fax: +353(0)1-402-2355

E-mail address: fjobrien@rcsi.ie

Abstract

In stem cell biology, focus has recently turned to the influence of the intrinsic properties of the extracellular matrix (ECM), such as structural, composition and elasticity, on stem cell differentiation. Utilising collagen-glycosaminoglycan (CG) scaffolds as an analogue of the ECM, this study set out to determine the effect of scaffold stiffness and composition on naive mesenchymal stem cell (MSC) differentiation in the absence of differentiation supplements. Dehydrothermal (DHT) and 1-ethyl-3-3-dimethyl aminopropyl carbodiimide (EDAC) crosslinking treatments were used to produce three homogenous CG scaffolds with the same composition but different stiffness values: 0.5, 1 and 1.5 kPa. In addition, the effect of scaffold composition on MSC differentiation was investigated by utilising two glycosaminoglycan (GAG) types: chondroitin sulphate (CS) and hyaluronic acid (HyA). Results demonstrated that scaffolds with the lowest stiffness (0.5 kPa) facilitated a significant up-regulation in SOX9 expression indicating that MSCs are directed towards a chondrogenic lineage in more compliant scaffolds. In contrast, the greatest level of RUNX2 expression was found in the stiffest scaffolds (1.5 kPa) indicating that MSCs are directed towards an osteogenic lineage in stiffer scaffolds. Furthermore, results demonstrated that the level of up-regulation of SOX9 was higher within the CHyA scaffolds in comparison to the CCS scaffolds indicating that hyaluronic acid further influences chondrogenic differentiation. In contrast, enhanced RUNX2 expression was observed in the CCS scaffolds in comparison to the CHyA scaffolds suggesting an osteogenic influence of chondroitin sulphate on MSC differentiation. In summary, this study demonstrates that, even in the absence of differentiation supplements, scaffold stiffness can direct the fate of MSCs, an effect that is further enhanced by the GAG type used within the CG scaffolds. These results have significant implications for the therapeutic uses of stem cells and enhance our understanding of the physical effects of the *in vivo* microenvironment on stem cell behaviour.

Introduction

In native tissues, cells are held within an extracellular matrix (ECM) which guides development and directs regeneration of the tissue. The ECM also serves to physically support the cells and provides them with environmental signals to direct cellular behaviour. However, the mechanical properties of native tissue or ECM can be as physically diverse as those of pliable brain tissue (1 kPa) to bone tissue (20 GPa). The goal of tissue engineering is to synthesise scaffolds that mimic the natural ECM to help guide the growth of new functional tissue *in vitro* or *in vivo* regardless of application.

Similarly to the natural ECM, scaffolds for tissue engineering have both a structural and functional role in tissue development. However, the distinction between them is becoming increasingly blurred as it has emerged over recent years that the structural properties have a modulatory effect on cell behaviour. Scaffold properties such as porous architecture, composition and degradation rates have been shown to have an influence on cell behaviour in terms of adhesion, proliferation and differentiation (Keogh *et al.* 2010, Murphy *et al.* 2010, Tierney *et al.* 2009, Zeltinger *et al.* 2001). More recently, scaffold mechanical properties or stiffness and the cell's response in terms of mechano-regulatory processes have begun to draw a lot of interest. Cell-ECM interactions, conducted via specific integrin-ligand adhesions, are accompanied by cytoskeletal action and matrix remodelling and contraction (Friedl *et al.* 1998). Whilst cell-mediated contraction is a natural phenomenon essential for wound healing (Yannas 2001), it was considered to have a disadvantageous effect on tissue engineered constructs. However, it has emerged that the mechanical input generated by the deformation of a substrate results in cellular mechano-transduction. Mechano-transduction is a process whereby cells transduce or convert physical force-induced signals into biochemical signals that are integrated into appropriate cellular responses (Huang *et al.* 2004, Ko *et al.* 2001). Not all anchorage-dependent cells respond to substrate stiffness in the same way; however mesenchymal stem cells (MSCs), fibroblasts and endothelial cells have demonstrated increased cell adhesion, spreading and proliferation on stiffer substrates. Cell migration has also shown to be influenced by stiffness gradients. Recently, Engler *et al* (2006) reported a key relationship between substrate stiffness and MSC differentiation whereby MSCs 'feel' or sense their surrounding matrix elasticity and transduce that information into morphological changes and lineage specification. However, the mechanism by which cells transduce these forces to biochemical signals is only beginning to be understood (Engler *et al.* 2006).

Collagen is a significant constituent of natural ECM and type I collagen is the primary structural collagen in mammalian tissues. Glycosaminoglycans (GAGs), another important component of the ECM, are linear polysaccharides containing repeating disaccharide units of an amino sugar. In tissue engineering scaffolds, addition of GAGs can stabilise the biomaterials and create an improved environment for cell activity (Pieper *et al.* 2000, Taylor *et al.* 2006, van Susante *et al.* 2001) e.g. polymerisation of collagen with chondroitin-6-sulphate has been shown to improve mechanical properties and biological activity of collagen (Yannas *et al.* 1975b, Yannas *et al.* 1975a). Collagen-glycosaminoglycan (CG) scaffolds are highly porous, biodegradable, bioactive scaffolds that are a well characterised template for tissue formation and have been used successfully, as a 3-D analogue of the ECM, in a range of tissue engineering applications including skin, nerve, cartilage and bone regeneration (Chamberlain *et al.* 1998, Lee *et al.* 2003, Lyons *et al.* 2010, Yannas 2001). The primary focus in our laboratory is the use of these scaffolds for bone and cartilage regeneration. Traditionally chondroitin sulphate (CS) is the GAG included within the CG scaffold as it is a key constituent of the bone and cartilage ECM. This collagen-chondroitin sulphate (CCS) scaffold has previously been shown to support osteogenesis and, to a lesser extent, chondrogenesis *in vitro* and *in vivo* (Farrell *et al.* 2006, Keogh *et al.* 2010, Lyons *et al.* 2010). However, one of the many advantages of the CG scaffold is the possibility of tailoring its composition for specific tissue engineering applications. HyA is a GAG that is more predominant in cartilage than bone ECM and has been shown to stimulate chondrogenesis *in vitro* and *in vivo* (Nehrer *et al.* 2006, van Susante *et al.* 2001, Wu *et al.* 2010). Subsequently HyA was hypothesised in this study to be more suitable than CS for cartilage tissue engineering.

Despite its potential for tissue regeneration, the CG scaffold is a compliant material with relatively weak mechanical properties and has demonstrated structural instability *in vitro* as a result of cell mediated contraction. However, it is possible to control scaffold stiffness via crosslinking (Haugh *et al.* 2011, Lee *et al.* 2001, Weadock *et al.* 1983). Crosslinking strengthens collagen-based biomaterials by introducing new bonds between the collagen molecules. Different crosslinking treatments exist that are typically grouped as either chemical or biophysical crosslinking. Dehydrothermal (DHT) treatment is a physical crosslinking treatment whereby the collagen is subjected to increased temperature (<98°C) while under vacuum. This removes any excess water within the collagen and forms intermolecular crosslinks between adjacent carboxylic and amine groups through a condensation reaction. 1-ethyl-3-3-dimethyl aminopropyl carbodiimide (EDAC) is a common chemical crosslinking agent that acts as a catalyst in the presence of *N*-Hydroxysuccinimide (NHS) to form bonds between the collagen fibres and GAGs within the biomaterial, producing urea as a by-product. However

this can be sufficiently removed by simple washing of the scaffold. One of the benefits of these two treatments is that these different crosslinking methods can be used to produce a range of CG scaffolds with the same composition but different compressive moduli. Compressive modulus has been shown to be the most relevant property when investigating cellular response to substrate properties (Engler *et al.* 2006, Harley *et al.* 2007).

To date a number of studies have been carried out to elucidate the effect of substrate stiffness on the differentiation of MSCs (Engler *et al.* 2006, Park *et al.* 2011, Tse *et al.* 2011); however, the majority of these studies have been carried out in 2-D or in hydrogels that do not necessarily mimic the native ECM. The objective of this study was to investigate if scaffold stiffness and composition effects the differentiation of naive MSCs. More specifically, the aims of this study were two-fold: utilising CG scaffolds and the crosslinking methods developed within our laboratory, this study set out to investigate the effect of scaffold stiffness on osteogenic and chondrogenic differentiation of naïve MSCs without the addition of differentiation supplements. Furthermore, based on the principle that changing the GAG type will alter cell behaviour, we sought to determine if MSC differentiation is further up-regulated towards a specific lineage depending on the GAG type.

Materials and Methods

Cell Culture

Primary MSCs, multipotent stem cells with the potential for both osteogenic and chondrogenic differentiation (Acosta *et al.* 2005, Pittenger *et al.* 1999), were isolated from the bone marrow of young male adult Wistar rats under ethical approval. MSCs were maintained in Dulbecco's Modified Eagles medium (DMEM) (Sigma-Aldrich, Dublin, Ireland) supplemented with 10 % foetal bovine serum (FBS), 1 % L-glutamine and 2 % penicillin/streptomycin, 0.5% Glutamax and 1% Non-essential amino acids (Biosciences, Dublin, Ireland) according to standard protocols established in our laboratory (Duffy *et al.* 2011). MSCs were used at passages 4-5 for all experiments.

Fabrication of CG Scaffolds

Scaffolds were produced by lyophilisation of a collagen-GAG suspension based on a previously developed protocol (Haugh *et al.* 2010, O'Brien *et al.* 2005). Two scaffold groups were used in the study: collagen-chondroitin sulphate (CCS) scaffolds and collagen-hyaluronic acid (CHyA) scaffolds.

CCS Scaffolds: A suspension was produced by blending micro-fibrillar bovine tendon-derived collagen (0.5 wt%) (Integra Life Sciences, Plainsboro, NJ) with chondroitin-6-sulphate (0.05 wt%), isolated from shark cartilage (Sigma-Aldrich, Dublin, Ireland) in 0.05 M acetic acid.

CHyA Scaffolds: A suspension was produced by blending fibrillar bovine tendon-derived collagen (0.5 wt%) with hyaluronic acid (0.05 wt%), sodium salt derived from streptococcus equi (Sigma-Aldrich, St. Louis, MO, USA,) in 0.5 M acetic acid. This higher concentration of acetic acid was used as HyA has a high anionic charge and a lower ph is required.

Both suspensions were maintained at 4°C during blending to prevent denaturation of the collagen. The suspension was lyophilised using a freezing process that was described previously. A final temperature of freezing of -10 °C was used in order to produce homogenous scaffolds with a mean pore size 325 µm (Haugh *et al.* 2010, Murphy *et al.* 2010).

Crosslinking

After freeze-drying, 12.7 mm diameter cylindrical samples were cut from the scaffold sheet. DHT treatment was carried out by placing the samples in an aluminium foil packet inside a vacuum oven (Vacucell 22; MMM) under a vacuum of 0.05 bar at temperature of 105 °C for 24 hr. These treatment conditions were found to increase stiffness with minimal denaturation of the collagen within the scaffold. DHT crosslinking also serves as a sterilisation method in order to prepare the scaffolds for cell culture (Haugh *et al.* 2011). Therefore this crosslinking treatment was carried out on all scaffold groups.

For EDAC crosslinking, two different concentrations of EDAC (Sigma-Aldrich, Germany) were used: 3 mM and 6 mM EDAC per gram CG scaffold. These concentrations were prepared in 2 ml of sterile distilled water and the samples were crosslinked in 24-well plates for 2 hr. N-Hydroxysuccinimide (NHS) is a catalyst that is commonly used with EDAC; a molar ratio of 2.5 M EDAC/M N-hydroxysuccinimide was used for all EDAC crosslinking. After crosslinking the scaffolds were washed twice using sterile PBS (Sigma-Aldrich). The concentrations of EDAC are expressed relative to a gram of collagen throughout this article.

Mechanical testing

Compressive testing was used to determine the modulus of the cell-free scaffolds to examine the effects of both crosslinking and GAG type. Mechanical testing of scaffold samples was carried out using a mechanical testing machine (Z050; Zwick/Roell) fitted with a 5-N load cell. Samples were prehydrated in PBS for 1 hr before testing and all testing was carried out in a bath of PBS. For unconfined compression testing with impermeable, un-lubricated platens, samples of 8mm diameter (n¼10 per group) were cut from the scaffolds using a punch. Testing was conducted at a strain rate of 10%/min. The modulus was defined as the slope of a linear fit to the stress–strain curve over 2%–5% strain.

Scaffold Seeding

The crosslinked scaffolds were placed in 6-well plates, one scaffold per well, and 200 µl of $1.25x10^6$ cell/ml MSC suspension was seeded on each side of the scaffold with a 30 min interval between each side. The seeded scaffolds were incubated for a further 30 mins and each well was then flooded with 5 ml of media. The un-seeded scaffolds were maintained in standard un-conditioned growth media (supplemented DMEM as

mentioned above). A partial feed was carried out every 3-4 days whereby 2.5 ml of media was replaced. Blebbistatin (50 μM, Sigma-Aldrich), a non-muscle myosin II (NM II) inhibitor that hinders the cells ability to sense matrix elasticity, was added to an additional group of scaffold cultures as a negative control. It was added to the media of the negative control group 24 hr post seeding. This was considered Day 0 for all the scaffold groups. Blebbistatin was replaced with every feed. All scaffold groups were maintained at 37°C with 5% CO_2 for 0, 1, 3, 7, 10 or 14 days before harvesting.

Gene Expression Analysis

Constructs (n = 3) were flash frozen in liquid nitrogen at each time point and stored at 80 C. RNA was isolated from the constructs by homogenisation in Qiazol lysis buffer (Qiagen, UK) using a rotor–stator homogeniser (Omni International, Marietta, GA). RNA was extracted using an RNeasy Mini Kit (Qiagen, UK) according to the manufacturer's instructions. RNA concentration was quantified using a spectrophotometer (absorption 260 nm). Following RNA extraction, trace genomic DNA was removed and the RNA sample was reverse transcribed using 200 ng of total RNA with an RT kit (QuantiTect RT Kit, Qiagen, UK) according to the manufacturer's instructions. Real-time polymerase chain reaction (PCR) was then carried out using a 7500 Real-time PCR System (Applied Biosystems, UK). The QuantiTect SYBR Green PCR Kit (Qiagen, UK) was used according to the manufacturer's instructions, with QuantiTect Primers (Qiagen, UK). Analysis of transcription marker expression was carried out. Specifically the contractile marker α-smooth muscle actin (SMA), transcription markers for osteogenesis (RUNX2) and chondrogenesis (SOX9), early osteogenic and chondrogenic markers alkaline phosphatase (ALP) and collagen type I (Col I), the mid stage osteogenic and chondrogenic markers, osteopontin (OPN) and collagen type II (Col II) were analysed. Expression levels were assessed using the relative quantification DDCt method. 18S acted as a housekeeping control.

Statistical Analysis

Two-way ANOVA followed by Holm-Sidak multiple comparisons was preformed to compare data. Error is reported in figures as the standard deviation SD and significance was determined using a probability value of $P<0.050$.

Results

Effect of Crosslinking on Compressive Modulus of Scaffolds

In order to determine if the GAG type would affect the resultant compressive modulus of the scaffolds we carried out compression testing on the crosslinked CCS and CHyA scaffolds. There was no significant difference between the different compositions (Fig 1A). As previously observed (Haugh *et al.* 2011), DHT crosslinking produced the most compliant scaffold with a compressive modulus of approximately 0.5 kPa in both CCS and CHyA scaffolds. Analysis of the mechanical testing of CCS and CHyA scaffolds after EDAC treatment revealed that compressive modulus increased with increasing EDAC concentration ($p<0.005$). 3 mM EDAC crosslinking imparted a stiffness value of approximately 1 kPa and a concentration of 6 mM EDAC produced the stiffest scaffold with a compressive modulus of approximately 1.5 kPa, representing a 3-fold increase in stiffness in comparison to the un-treated (DHT) scaffolds.

Cells use traction forces to probe their physical environments and SMA expression has been shown to enhance cell traction forces in response to increasing substrate stiffness (Chen *et al.* 2007). In order to determine if MSCs could distinguish between the scaffolds with different stiffnesses, we investigated the levels of SMA expression in all scaffold groups. A significant increase in SMA expression was observed with increasing compressive modulus 24 hr post-seeding (Fig. 1B). The most compliant scaffold, with a compressive modulus of 0.5 kPa, had significantly lower levels of SMA expression in comparison to the other scaffold groups. There was a significant increase in fold expression as the compressive modulus increased with the stiffest scaffolds of 1.5kPa compressive modulus demonstrating the highest levels of expression of SMA indicating that the cells are exerting greater traction forces on the scaffold due to the increase in stiffness. However, the differences observed in expression were confined to scaffold stiffness and not composition. There was no difference in SMA expression between the CCS and CHyA scaffolds.

Effect of Scaffold Stiffness and Composition on MSC Differentiation

Real time PCR was carried out at all time points to determine the expression levels of early transcription markers for osteogenesis (RUNX2) and chondrogenesis (SOX9) across the range of scaffold stiffnesses for both scaffold compositions (Fig. 2). The stiffest of the three scaffold groups (1.5 kPa) supported a significant up-regulation of RUNX2 expression in comparison to the less stiff scaffolds. This was observed in both the CCS scaffolds and CHyA scaffolds (Fig. 2A). Within the CCS scaffolds, RUNX2 expression increased

5-fold at day 3 relative to MSCs alone in standard 2-D culture, but this had decreased to a 0.5 fold increase by day 7. A 4-fold increase, relative to MSCs alone in standard 2-D culture, was observed in RUNX2 expression in CCS scaffolds with a compressive modulus of 1 kPa at day 7. However, this was significantly lower than the up-regulation observed in the stiffest CCS scaffold (1.5 kPa) at day 3 ($P<0.001$). A 3-fold increase in RUNX2 expression relative to MSCs alone was observed in the stiffest CHyA scaffold (1.5 kPa), however the level of expression observed in the CCS scaffolds was significantly higher than observed in the CHyA scaffolds. Conversely, in comparison to RUNX2 expression, an up-regulation of SOX9 expression was observed in the most compliant CCS scaffolds (0.5 kPa) in comparison to the other scaffold variants in both scaffold compositions (Fig. 2B). A significant increase was observed at day 10 whereby a 5 fold increase in expression was observed relative to MSCs alone in standard 2-D culture. Up-regulation of SOX9 was observed in all the CHyA scaffolds by day 3. However, similarly to the CCS scaffolds, the most compliant scaffold (0.5 kPa) demonstrated a significant increase in SOX9 expression in comparison to the other scaffold stiffnesses. In addition, this increase was significantly higher and occurred at an earlier time point in CHyA scaffolds in comparison to the CCS scaffolds.

To fully elucidate the effect of GAG type on MSC differentiation, we extracted the results of specific interest from Fig. 2 and compared RUNX2 expression in the stiffest of both scaffold compositions (1.5 kPa) and SOX9 expression within the most compliant of the two scaffold compositions (0.5 kPa) (Fig. 3). As mentioned earlier, both of the stiffest variants of each scaffold composition (1.5 kPa) demonstrated significant up-regulation of RUNX2 expression in comparison to the less stiff scaffolds. A significant increase in expression was observed at one time point only in both scaffold types; day 3 in the CCS scaffold and day 10 in the CHyA scaffold. However, when directly compared, the difference in expression levels between the two different scaffold compositions is more apparent (Fig. 3A). The CCS scaffold facilitated significantly higher RUNX2 expression in comparison to the CHyA scaffold. Additionally, the peak in RUNX2 expression emerged at an earlier time point (day 3) within the CCS scaffolds in comparison to day 10 in the CHyA scaffolds. SOX9 expression was significantly higher in the scaffolds with a compressive modulus of 0.5 kPa (Fig. 3B). This was consistent for both scaffold compositions. However, when presented side by side, a noticeable difference in the expression trend over the culture period emerged. The most compliant of the CCS scaffolds maintained a consistent level of expression up to day 10, at which point a significant up-regulation in SOX9 expression occurred whereby a 5 fold increase in expression was observed before dropping off again at day 14. Within the CHyA scaffolds, SOX9 expression was observed at day 0 and day 1 but an up-regulation was observed at day 3

whereby a 9 fold increase in expression was observed. This increase occurred at an earlier time point and was significantly higher to the increase observed in the CCS scaffolds. However similarly to CCS scaffolds, SOX9 expression within the CHyA scaffolds had decreased to a 1 fold increase in expression by day 7.

Effect of Non-Muscle Myosin II on MSC Differentiation

In order to determine if the lineage specification observed in both scaffold types was a direct effect of substrate stiffness we treated the cells with blebbistatin, a potent NM II inhibitor that hinders the cells ability to sense the elasticity of the matrix and thus prevents mechano-transduction. The influence of substrate stiffness on cellular mechano-transduction was apparent when un-treated and blebbistatin treated scaffolds were shown side by side. The un-treated scaffold groups were the scaffolds that previously demonstrated the most significant changes in transcription marker expression in the absence of differentiation supplements. The blebbistatin groups are the same scaffold variants and corresponding time-points but the cells within the scaffolds have been treated with blebbistatin. The highest levels of RUNX2 expression was observed in CCS scaffolds (1.5 kPa) at day 3 and in the CHyA scaffolds (1.5 kPa) at day 10. However when these scaffolds were cultured in blebbistatin-treated media, there was no up-regulation of RUNX2 within the scaffolds (Fig. 4A). Similarly, an increase in SOX9 expression was observed in CCS scaffolds (0.5 kPa) at day 10 and CHyA scaffolds (0.5 kPa) at day 3 when cultured in standard growth media (Fig. 4B), but the introduction of blebbistatin into the media resulted in no expression. These results demonstrate that the up-regulation in transcription markers observed was a direct result of the cells sensing the elasticity of the scaffold.

Expression of Differentiation Markers

No changes in expression of Col I, Col II or ALP were seen as a result of altered stiffness or GAG type in any of the scaffolds at any time point. However a small but significant increase in OPN, a mid/late stage marker of osteogenic differentiation, was observed in the stiffest CCS scaffolds at day 7 and day 10 but expression levels were negligible within the CHyA scaffolds at all time points (Fig. 5).

Discussion

In recent years, the rigidity of a substrate and the mechanical input generated by its deformation has been shown to regulate adult stem cell differentiation. Engler *et al* (2006) has provided striking evidence that matrix elasticity influences higher cellular functions such as stem cell differentiation whereby matrix rigidity and actomyosin contractility modulated MSC differentiation (Discher *et al.* 2005, Engler *et al.* 2006, Zajac *et al.* 2008). However, many of these investigations have been carried out on 2-D surfaces or in hydrogels which do not necessarily mimic the native ECM (Dado *et al.* 2009, Engler *et al.* 2006, Rehfeldt *et al.* 2007). The rapidly increasing use of 3-D scaffolds in tissue engineering requires a better understanding of the significant role of scaffold micro-architecture and mechanical properties in influencing cell behaviour and overall bioactivity. This study utilised CG scaffolds to determine the correlation between scaffold stiffness, composition and naïve MSC differentiation. Scaffolds with the lowest stiffness (0.5 kPa) demonstrated a significant up-regulation in SOX9 expression indicating that MSCs are directed towards a chondrogenic lineage within the more compliant scaffolds. The level of up-regulation of SOX9 was higher within the CHyA scaffolds in comparison to the CCS scaffolds indicating that hyaluronic acid further influences chondrogenic differentiation. In contrast, the greatest level of RUNX2 expression in the stiffest scaffolds (1.5 kPa) indicated that MSCs are directed towards an osteogenic lineage within these scaffolds. Enhanced RUNX2 expression was observed in the CCS scaffolds in comparison to the CHyA scaffolds suggesting an osteogenic influence of chondroitin sulphate on MSC differentiation. These results were observed in the absence of any differentiation supplements indicating that the effect observed was due to the stiffness of the scaffold sensed by the cells and that this is further enhanced by the GAG type used within the CG scaffolds.

In tissue engineering, 3-D scaffolds are used extensively as analogues of native ECM and the CG scaffold is an attractive candidate as it has well-characterised and controllable mechanical, compositional and micro-structural properties (Haugh *et al.* 2010, Murphy *et al.* 2010, Murphy *et al.* 2010, O'Brien *et al.* 2004, O'Brien *et al.* 2005, Tierney *et al.* 2009). We recently utilised two crosslinking methods, DHT and EDAC, to enhance the mechanical properties of the CG scaffolds, producing scaffolds with a range of stiffness values (Haugh *et al.* 2011), and from this three crosslinking groups were selected for the purposes of this study; DHT, 3 mM EDAC and 6 mM EDAC. This resulted in CCS scaffolds with three different moduli; 0.5, 1 and 1.5 kPa. Applying the same crosslinking treatments to the CHyA scaffolds imparted a modulus range comparable to the CCS scaffolds (Fig 1A) whereby DHT crosslinking resulted in the most compliant of the scaffolds while 3 mM

EDAC and 6 mM groups demonstrated a 2-fold and 3-fold increase in compressive modulus respectively. The mechanical properties of different native tissues are very diverse and can vary, for example, from soft brain tissue (0.1 kPa) to pre calcified bone (100 kPa) to rigid compact bone (20GPa) (Fig. 6A); the range of scaffold stiffnesses analysed in this study is significantly limited by comparison. However, even with this limited range and in the absence of supplementation, it is interesting to note the significant influence of scaffold stiffness on the lineage specification of the MSCs demonstrating just how mechano-sensitive the cells are to changes in their environment.

Integrins provide a mechanical link between the ECM and the actomyosin cytoskeleton of cells. Contractile forces are generated by the ubiquitous crossbridging interactions of actin and myosin-II filaments in stress fibres. These forces are transmitted to the substrate as traction forces. Focal adhesions link matrix-attached transmembrane integrin receptors to the actin cytoskeleton via a complex of anchorage proteins (Fig. 6B). The generation of contractile forces is strongly associated with the expression of SMA which is enriched in stress fibres and focal adhesion sites. Force transmission between actin filaments and focal adhesions may be enhanced through the incorporation of SMA into actin filaments (Dugina *et al.* 2001). SMA has been shown to up-regulate cell traction forces and enhance the formation of stress fibres within the actomyosin (Chen *et al.* 2007). Cells can discriminate subtle changes in the scaffold micro-architecture that may affect their behaviour and within our scaffold groups, expression of SMA increased with increasing compressive modulus indicating that the MSCs were re-organising their cytoskeleton. This suggests that the cells were able to distinguish a change in elasticity across the stiffness range and exert greater traction forces in response to the stiffness of the substrate increasing. However, there was no difference in SMA expression between the two scaffold compositions. This result validates the premise of the study as it demonstrates that SMA expression is stiffness dependent and is not influenced by GAG type (Fig. 2).

Attention has been increasingly drawn to the structural properties of the ECM as a stimulant for stem cell differentiation. The composition of the matrix can influence cell behaviour via differential integrin binding to adhesion ligands, and as already discussed the mechanical properties of the matrix can influence cell differentiation via mechano-transduction. In order to determine if the structural properties of the CG scaffold would influence MSC differentiation, without the presence of any soluble biochemical factors, we investigated the expression of RUNX2 and SOX9 (Fig. 3). RUNX2 is a transcription factor that is required for commitment of mesenchymal progenitor cells to the osteoblast lineage (Franceschi *et al.* 2003, Karsenty *et al.* 1999). SOX9

is a transcription factor that is expressed in all chondro-progenitor cells and differentiated chondrocytes (Ng *et al.* 1997). Within the stiffness range analysed, the stiffest of both the CCS and CHyA scaffolds (1.5 kPa) supported a significant up-regulation of RUNX2 (osteogenic marker) expression in comparison to the less stiff 0.5 kPa and 1 kPa scaffolds demonstrating that stiffer scaffolds are more likely to induce MSCs differentiation down an osteogenic lineage. Conversely higher levels of SOX9 expression was seen in the most compliant scaffolds (0.5 kPa) demonstrating that these scaffolds are more likely to induce MSCs differentiation down a chondrogenic lineage.

An important point to note about the mechanical properties referred to in this study is that the stiffness of the individual CG struts to which the cells attach differs by orders of magnitude from that of the bulk mechanical properties of the CG scaffolds. For example, it was previously found that the individual strut modulus of a similar collagen-GAG (~5MPa) scaffold was in the order of 2.5×10^3 times higher than the bulk modulus of the scaffold (~200Pa) (Harley *et al.* 2007). However, unlike in scaffolds where the mean pore size greatly exceeds the dimension of the cells and effectively the cell is presented with a planar or 2-D surface, in scaffolds with smaller mean pore sizes, the cells may bridge across the pores and attach to two or more struts. Therefore, the surface that the cells adhere to is no longer simply 2-D in orientation. We have previously shown that this occurs in the collagen-GAG scaffolds used in this study (Jungreuthmayer *et al.* 2009a) and that this 3-D mode of attachment greatly affects cell behavior (Jungreuthmayer *et al.* 2009b). For this reason, we suggest that the bulk scaffold mechanical properties referred to in this study are particularly valid since they give a representation of the mechanical properties of the 3-D structure. However, we acknowledge that a caveat with the current study is that we have not presented data demonstrating how the three crosslinking treatments used affects the scaffold strut modulus. This is not a trivial analysis but is nonetheless something we will be investigating in the future.

While this data successfully demonstrates the intrinsic function of ECM elasticity, the ECM composition also imparts a significant influence on cell behaviour (Martino *et al.* 2009, Reilly *et al.* 2010). The composition of CG scaffolds has been shown to have an effect on cell behaviour and can be altered for specific applications by altering the GAG type used (Tierney *et al.* 2009). Traditionally CS is used as the GAG component in the development of CG scaffolds. CS containing scaffolds have previously demonstrated higher levels of osteogenic differentiation and the CCS scaffolds have been successfully used both *in vitro* and *in vivo* for bone tissue engineering (Lyons *et al.* 2010, Tierney *et al.* 2009, Wollenweber *et al.* 2006). However, we

incorporated HyA into the scaffolds in place of CS as it has been shown to be more chondro-inductive than CS and therefore more suitable for cartilage tissue engineering (Banu *et al.* 2007). The results demonstrated that the GAG type used within the CG scaffolds had a significant impact on the level of differentiation observed within the CG scaffolds (Fig. 4). The stiffest scaffolds (1.5 kPa) exhibited the highest levels of RUNX2 expression in comparison to the less stiff scaffolds within the stiffness range (Fig. 4A). However, within the 1.5 kPa stiffness group, the CCS scaffolds facilitated a significantly higher level of RUNX2 expression in comparison to the CHyA scaffolds. This up-regulation of RUNX2 was observed at day 3 in CCS scaffolds in comparison to day 14 in CHyA scaffolds indicating an earlier onset of differentiation within the CCS scaffolds. These results suggest that CS has a greater osteogenic influence on MSC differentiation. Scaffolds with the lowest stiffness of 0.5 kPa demonstrated the highest levels of SOX9 expression (Fig. 4B); however within the 0.5 kPa stiffness group, significantly enhanced expression of SOX9 was observed within the CHyA scaffolds in comparison to the CCS scaffolds. Furthermore SOX9 expression was observed 7 days earlier within the CHyA scaffolds indicating that hyaluronic acid further influences chondrogenic differentiation of MSCs.

Aside from the influence of scaffold micro-structural properties, cell activity, such as adhesion and migration, is a contributing factor to the effect matrix elasticity has on stem cell differentiation. Anchorage-dependent cells exert traction forces that enable the cells to probe and sense their surrounding matrix. These traction forces increase as substrate stiffness increases. The ability of the cell to generate increasing levels of tension based on substrate stiffness is regulated by myosin motor proteins which generate traction forces against the matrix. NM II is an actin-binding myosin that has actin crossbridging and contractility properties. NM II is an integral part of cellular response to mechanical stimulation that can be inhibited by pharmacological treatments, such as blebbistatin, that ultimately block differentiation (Engler *et al.* 2006, Krieg *et al.* 2008). The influence of substrate stiffness on cellular mechano-transduction was apparent when the scaffolds, which were cultured in normal growth media, were compared to scaffolds treated with blebbistatin for RUNX2 and SOX9 expression at the same time points at which the highest levels of expression of each transcription marker was previously observed. There was no increase in RUNX2 (Fig. 4A) or SOX9 (Fig. 4B) expression in the blebbistatin-treated scaffolds demonstrating that the lineage specification observed was a direct result of matrix-elasticity sensing by the MSCs.

Currently, the majority of studies investigating the effect of substrate stiffness have utilised biochemical factors to terminally differentiate the cells. However, we sought to determine if the structural

properties of the scaffold alone could terminally commit the MSCs to a specific lineage. Due to the increased expression of RUNX2 seen in the stiffer scaffolds group, we analysed the expression of early (Col I and ALP) and mid/late (OPN) markers of osteogenic differentiation. There was no significant increase in Col I or ALP expression in any of the CCS or CHyA scaffolds; however, a small but statistically significant increase in OPN expression was observed within the stiffest CCS scaffold in comparison to all other scaffolds including, interestingly, the stiffest CHyA scaffold (Fig. 5). This suggests that this up-regulation in osteopontin was due to the combined effect of increased stiffness and the presence of chondroitin sulphate. When chondrogenic differentiation was analysed by looking at Col II expression, no significant changes were seen. These results suggest that, although MSC fate is directed towards specific lineages, further stimulation with conditioned media (osteogenic and chondrogenic supplementation during culture) is required for the MSCs to fully differentiate down each lineage. A further limitation of this study was the failure to definitively determine the fate of the MSCs. This study has demonstrated an early response in MSCs that is suggestive of their lineage commitment. Ultimately, to fully understand the influence of scaffold stiffness on MSC differentiation, a longer term study with supplementation is required.

Conclusion

This study has demonstrated that scaffold micro-architecture has a significant influence on the fate of MSCs whereby the stiffest scaffolds directed MSCs towards an osteogenic lineage and the most compliant of the scaffolds directed MSCs towards a chondrogenic lineage. Furthermore, the study demonstrates that the type of glycosaminoglycan used in the scaffolds also enhances the lineage specification of MSCs, whereby hyaluronic acid enhanced chondrogenic differentiation and chondroitin sulphate enhanced osteogenic MSC differentiation. These results were observed in the absence of any differentiation supplementation and demonstrate the possibility of tailoring the intrinsic scaffold properties to control stem cell differentiation for specific tissue engineering applications.

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Figure Captions:

Figure 1: (A) Comparison of the compressive modulus of CCS scaffolds and CHyA scaffolds after crosslinking via DHT, 3mM and 6 mM EDAC crosslinking. *P<0.001 relative to DHT crosslinked scaffolds. ** P<0.005 relative to both DHT and 3 mM EDAC crosslinked scaffolds. **(B)** SMA expression in both CCS scaffolds and CHyA scaffolds across the stiffness range. * P<0.001 relative to 0.5 kPa scaffold. ** P< 00.05 relative to both 0.5 kPa and 1 kPa scaffolds. There was no significant difference between the CCS and CHyA scaffolds.

Figure 2: (A) Expression of osteogenic (RUNX2) transcription marker in both CCS scaffold and CHyA scaffold across the stiffness range. * P<0.001 relative to all other stiffness variants at all time points. ** P <0.001 relative to 0.5 kPa scaffold. **(B)** Expression of chondrogenic (SOX9) transcription marker in both CCS scaffold and CHyA scaffold across the stiffness range. * P<0.005 relative to other stiffness variants at all time points.

Figure 3: (A) Comparison of RUNX2 expression in CCS scaffolds and CHyA scaffolds with a compressive modulus of 1.5 kPa. * P<0.001 relative to CHyA scaffold and relative to all time points in both scaffold variants. ** P < 0.005 relative to all time points for CHyA scaffold. **(B)** SOX9 expression in both CCS scaffold and CHyA scaffold with a compressive modulus of 0.5 kPa. *P<0.001 relative to CCS scaffold and relative to all time points in both scaffold variants. ** P<0.005 relative to all time points for CCS scaffold.

Figure 4: The comparison of RUNX2 **(A)** and SOX9 **(B)** expression in scaffolds cultured in growth media versus blebbistatin treated media.* P<0.001 relative to the blebbistatin treated scaffolds.

Figure 5: Comparison of the expression of mid/late stage osteogenic marker OPNwithin the stiffest of the scaffold variants (1.5 kPa) in both CCS scaffolds and CHyA scaffolds. * P<0.005 relative to CHyA scaffold

Figure 6: (A) Range of elasticity and relative tissue types. **(B)** Illustration depicting the attachment of a cell to a scaffold and the resultant cytoskeletal formations. Both illustrations adapted from Zajac et al., (2008).

Figure 1:

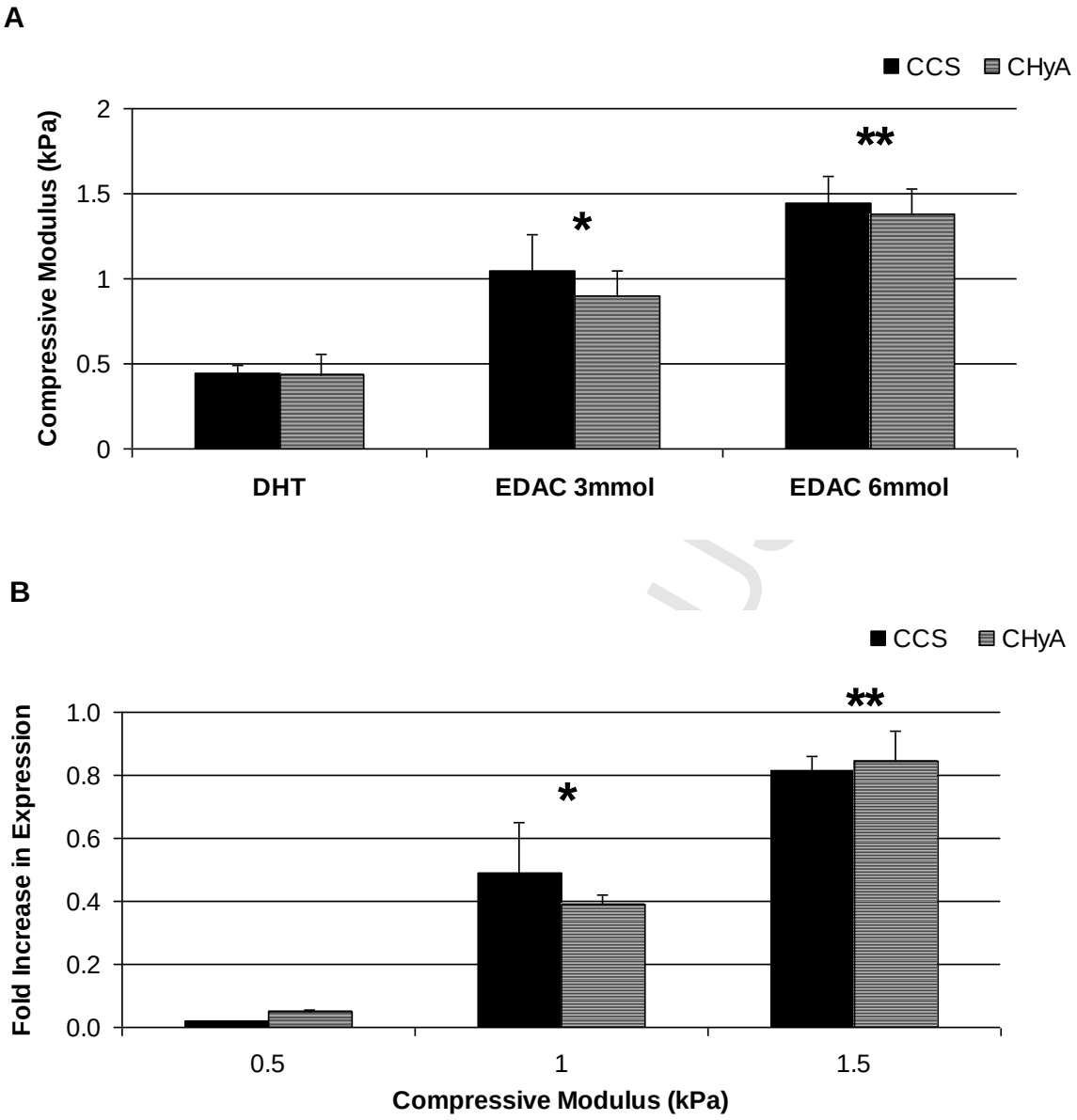
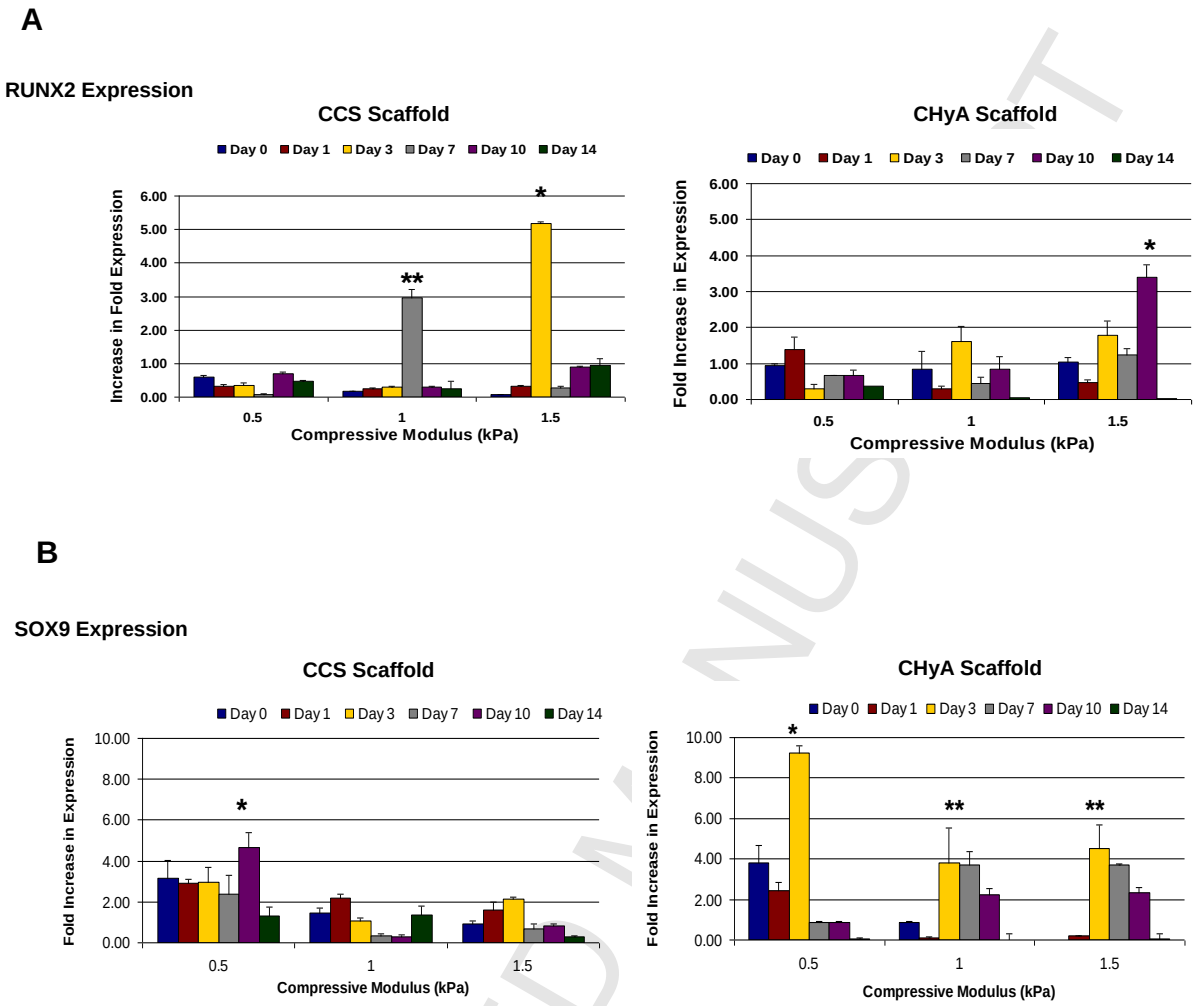


Figure 2:



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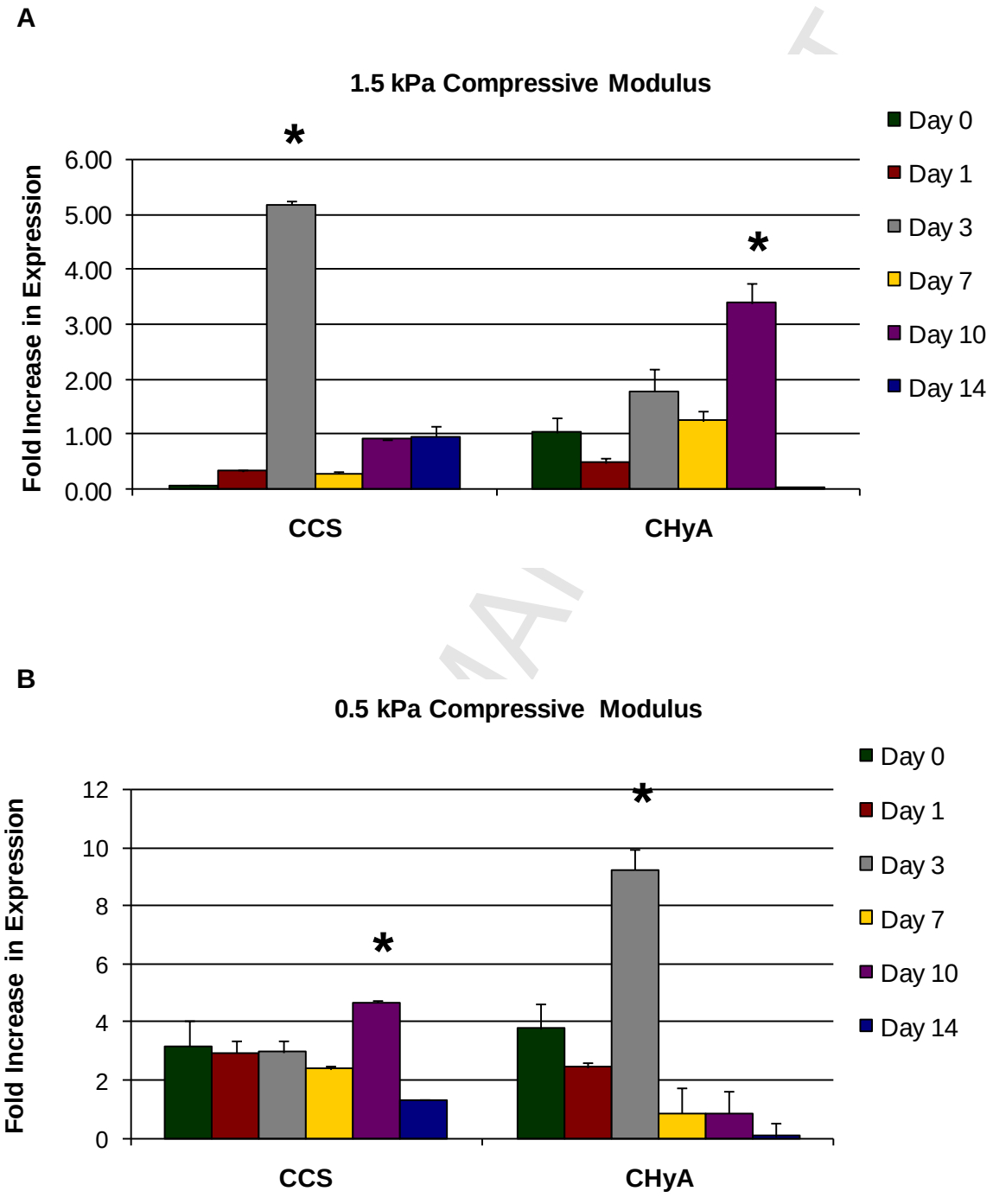

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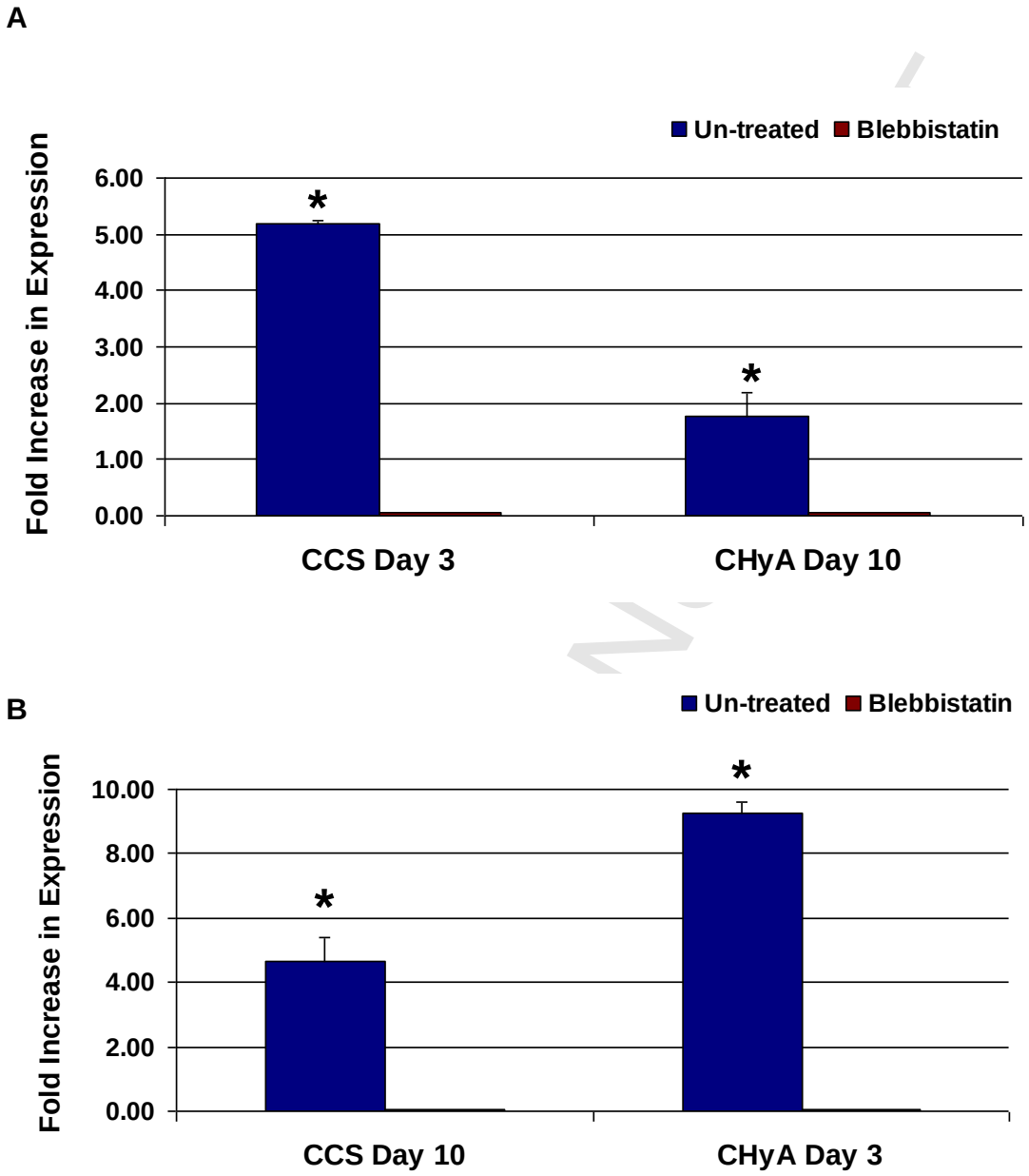

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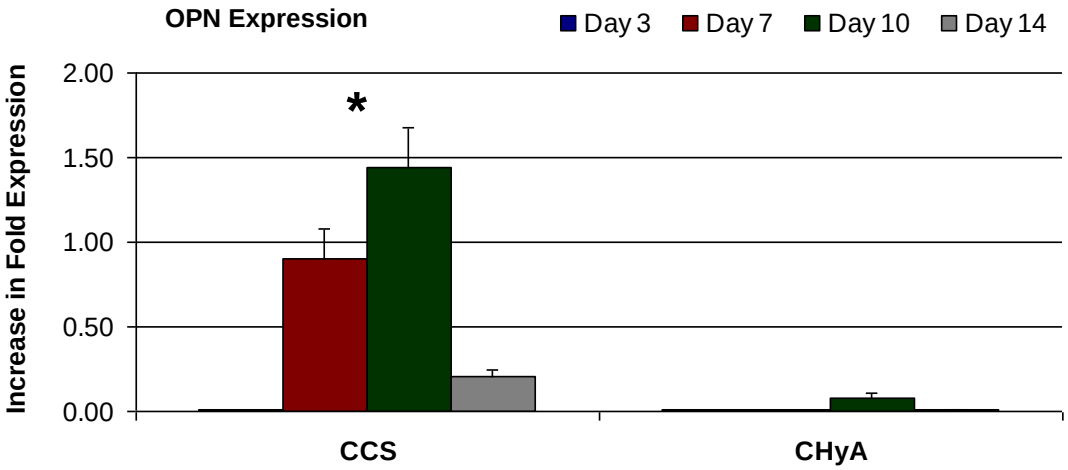

Figure 6: