Low oxygen tension is a more potent promoter of chondrogenic differentiation than dynamic compression

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Abstract:

During fracture healing and microfracture treatment of cartilage defects mesenchymal stem cells (MSCs) infiltrate the wound site, proliferate extensively and differentiate along a cartilaginous or an osteogenic lineage in response to local environmental cues. MSCs may be able to directly sense their mechanical environment or alternatively, the mechanical environment could act indirectly to regulate MSC differentiation by inhibiting angiogenesis and diminishing the supply of oxygen and other regulatory factors. Dynamic compression has been shown to regulate chondrogenesis of MSCs. In addition, previous studies have shown that a low oxygen environment promotes *in vitro* chondrogenesis of MSCs. The hypothesis of this study is that a low oxygen environment is a more potent promoter of chondrogenic differentiation of MSCs embedded in agarose hydrogels compared to dynamic compression. In MSC-seeded constructs supplemented with TGF-β3, GAG and collagen accumulation was higher in low oxygen conditions compared to normoxia. For normoxic and low oxygen culture GAG accumulation within the agarose hydrogel was inhomogeneous, with low levels of GAG measured in the annulus of constructs maintained in normoxic conditions. Dynamic compression did not significantly increase GAG or collagen accumulation in normoxia. However under low oxygen conditions, dynamic compression reduced GAG accumulation compared to free-swelling controls, but remained higher than comparable constructs maintained in normoxic conditions. This study demonstrates that continuous exposure to low oxygen tension is a more potent pro-chondrogenic stimulus than one hr/day of dynamic compression for porcine MSCs embedded in agarose hydrogels.

1. Introduction:

Mesenchymal stem cells (MSCs) are multipotent progenitor cells found in various tissues of adults that have the ability to proliferate extensively while maintaining their multipotent differentiation capabilities (Kadiyala et al., 1997; Bruder et al., 1997; Pittenger et al., 2001). During fracture healing and microfracture treatment of cartilage defects, MSCs from the bone marrow and other surrounding soft tissues infiltrate the wound site, proliferate extensively and differentiate along a cartilaginous or an osteogenic lineage in response to local environmental cues such as growth factors and cytokines. The mechanical environment is also known to regulate the mechanisms of repair following bone fracture. When there is excess motion at the site of injury the predominant mechanism of bone regeneration is through endochondral ossification. Conversely, when motion is minimized, healing primarily occurs through intramembranous ossification. A number of different hypotheses have been proposed to explain how the mechanical environment regulates endochondral ossification during fracture healing. MSCs may be able to *directly* sense their mechanical environment within a regenerating tissue and differentiate based on the local magnitude of shear strain, hydrostatic pressure, tensile strain, compressive strain and/or fluid flow they experience (Pauwels 1980; Prendergast et al., 1997; Carter et al., 1998; Claes and Heigele, 1999; Garcia et al., 2002; Lacroix and Prendergast, 2002, Kelly and Prendergast, 2005). In conjunction or perhaps alternatively, the mechanical environment could also act *indirectly* to regulate MSC differentiation by inhibiting angiogenesis and hence the supply of oxygen and other factors to the wound site (Ferguson et al., 1999).

Uncoupling the different roles the mechanical environment can have on endochondral ossification during fracture healing has proved challenging using *in vivo* and *in silico* models. Such studies have demonstrated that fracture healing is strongly influenced by mechanical

factors, such as loading, fixation stiffness and gap size (Choe et al., 1998; Goodship and Cunningham, 2001; Lacroix and Prendergast, 2002). Pauwels (1980) proposed that stress and strain invariants guide the differentiation pathway, whereby hydrostatic pressure results in cartilage formation; while distortional strain, or elongation, favored fibrous tissue formation. A similar concept has been adopted by Carter et al. (1998) to explain a number of mechanobiological processes (e.g. Carter and Wong, 2003; Carter et al., 2004). Further developing this concept, fluid flow and shear strain have also been hypothesized as the stimuli that direct tissue differentiation over time (Prendergast et al., 1997). These biophysical stimuli may directly act to regulate differentiation, or they may interact with biological factors such as growth factors or cytokines. A number of studies have suggested that the mechanical environment during fracture healing can also regulate the neo-vascularization necessary for new bone formation (Geris et al., 2003; Shefelbine et al., 2005). The hypoxic environment generated by poor vascularity has been shown to promote a more chondrogenic rather than an osteogenic phenotype (Hirao et al., 2006). More recently, computational models of tissue differentiation have been extended to include factors such as angiogenesis (Checa and Prendergast, 2009), providing further support for the hypothesis that capillary formation is indeed mechano-regulated. However, the relative roles played by the local oxygen tension and mechanical signals in regulating endochondral ossification have yet to be elucidated.

A number of *in vitro* models have been developed, primarily in the field of tissue engineering, to investigate the roles of environmental factors such as mechanical signals and oxygen tension on the differentiation pathway of MSCs. For example, dynamic compression has been shown to regulate chondrogenesis of bone marrow derived MSCs encapsulated in agarose gel. (Angele et al., 2004; Huang et al, 2004; Campbell et al., 2006; Mauck et al., 2007 ;

Terraciano et al., 2007; Thorpe et al., 2008; Kisiday et al., 2009; Huang et al., 2010; Thorpe et al., 2010). Similar studies have shown that a low oxygen environment promotes chondrogenesis of MSCs (Lennon et al., 2001; Schere et al., 2004; Robbins et al., 2005; Wang et al., 2005; Krimmer et al., 2009; Kanichai et al., 2008). As already described, the mechanical environment within a fracture callus can promote endochondral ossification in one of two ways. Firstly, the biophysical stimuli that act on the MSCs within the defect could directly promote differentiation along the chondrogenic pathway. Secondly, by preventing neo-vascularization and promoting a local hypoxic environment, mechanical loading could indirectly promote chondrogenesis of MSCs. The objective of this study is to determine the relative roles of mechanical signals and oxygen tension on chondrogenesis of bone marrow (BM) derived MSCs using a well developed tissue engineering model. We hypothesized that a low oxygen environment is a more potent promoter of chondrogenic differentiation of MSCs compared to dynamic compression.

2. Methods:

2.1 Cell isolation and construct assembly

Bone marrow was aseptically harvested from the femoral diaphysis of four month old pigs (~50 kg). Porcine MSCs were isolated and expanded according to a modified method developed for human MSCs (Lennon and Caplan, 2006). Cells were seeded at a density of 50,000 cells/cm^2 in T-75 cm^2 flasks in high-glucose DMEM GlutaMAX supplemented with 10% foetal bovine serum and 100 U/mL penicillin/streptomycin (all GIBCO, Biosciences, Dublin, Ireland). MSCs were subcultured at a ratio of 1:3 following colony formation and expanded to passage three. Cells were suspended in 2% agarose (Type VII, Sigma-Aldrich, Arklow, Ireland) at a density of 15 million cells/ml and cast in a stainless steel mould to produce cylindrical

constructs (Ø5 mm x 3 mm thickness). Constructs were exposed to either normal (21%) or low oxygen (5%) tensions for six weeks. Agarose hydrogel constructs were maintained in a chemically defined chondrogenic medium (CDM) consisting of DMEM GlutaMAX™ supplemented with penicillin (100 U/mL)-streptomycin (100 μg/mL) (both GIBCO, Biosciences, Ireland), 100 μg/ml sodium pyruvate, 40 μg/ml L-proline, 50 μg/ml L-ascorbic acid-2-phosphate, 1.5 mg/ml BSA, 1× insulin–transferrin–selenium, 100 nM dexamethasone (all from Sigma-Aldrich, Ireland) with or without 10 ng/ml recombinant human transforming growth factor-β3 supplementation (TGF-β3; ProSpec-Tany TechnoGene Ltd, Israel). Medium was changed twice per week.

2.2 Dynamic compression application

Intermittent dynamic compression was carried out in custom developed compressive loading bioreactors placed within the normal and low oxygen incubators. The dynamic compression protocol consisted of 10% strain amplitude superimposed on a 0.01 N preload at a frequency of 1 Hz. This loading regime was employed for a period of 1 hour/day, 5 days/week. Continuous dynamic compression (CDC) constructs were loaded for six weeks, while delayed dynamic compression (DDC) constructs were only loaded during weeks 4-6 (Fig. 1). Free swelling (FS) constructs were maintained in parallel culture conditions as controls.

2.3 Mechanical analysis

Constructs were assessed at days 0, 21, and 42. Three constructs from each group were mechanically tested in unconfined compression between impermeable platens using a standard materials testing machine with a 5N load cell (Zwick Z005, Roell, Germany). Stress relaxation

tests were performed, consisting of a ramp and hold cycle with a ramp displacement of 1 μm/s until 10% strain was obtained and held until equilibrium was reached (30 min). Dynamic tests were performed immediately after the stress relaxation cycle. A cyclic strain amplitude of 1% superimposed upon the 10% strain was applied for 10 cycles at 1 Hz. The compressive equilibrium modulus and dynamic modulus were determined from these tests.

2.4 Quantitative biochemical analysis

Following mechanical testing, the constructs were cored using a 3 mm biopsy punch, the wet mass of the annulus and core was recorded and frozen for subsequent biochemical analyses. Annulus and core samples were digested with 125 μg/mL papain in 0.1 M sodium acetate, 5 mM L-cysteine-HCl, 0.05 M EDTA, pH 6.0 (all from Sigma-Aldrich) at 60 °C under constant rotation for 18 h. DNA content was quantified using the Hoechst Bisbenzimide 33258 dye assay as described previously (Kim et al., 1988), with a calf thymus DNA standard. Sulphated glycosaminoglycan (sGAG) content was quantified using the dimethylmethylene blue dye-binding assay (Blyscan, Biocolor Ltd., Northern Ireland), with a chondroitin sulphate standard. Total collagen content was determined by measuring the hydroxyproline content. Samples were hydrolysed at 110 °C for 18 hrs in 38% HCl and assayed using a chloromine-T assay (Kafienah and Sims, 2004) with a hydroxyproline:collagen ratio of 1:7.69 (Ignat'eva et al., 2007). Each biochemical constituent was normalised to the tissue wet weight.

2.5 Histology and immunohistochemistry

At each time point, two additional samples per group were fixed in 4% paraformaldehyde overnight, dehydrated, and embedded in paraffin. Constructs were sectioned to 6 μm thickness

perpendicular to the disc face. Sections were stained with 1% alcian blue 8GX (Sigma-Aldrich) in 0.1 M HCl for sGAG, and picro-sirius red for collagen. Type I and II collagen were identified through immunohistochemistry (Buckley et al., 2010). Negative and positive controls of porcine ligament and cartilage were included.

2.4 Statistical analysis

Mechanical and biochemical properties are expressed in the form of mean ± standard deviation, with n = 3 for each group at each time point. All statistical analysis was performed using one-way ANOVA with Tukey post-hoc tests to enable comparisons between groups. A level of $p<0.05$ was considered significant.

3. Results:

At day 42, the DNA content was lower compared to the initial values at day 0 (Fig. 2A). GAG and collagen accumulation increased with time in culture for constructs supplemented with TGF-β3, but for growth factor-free constructs there were no significant increases in the biochemical content (Fig. 2B-D). In the TGF-β3 supplemented groups the GAG and collagen accumulation, as a percentage of the wet weight (%W.W.) or when normalized by DNA content, were significantly higher in low oxygen compared to normoxic conditions. These differences between normoxic and low oxygen environments were observed in both the core and annular regions of constructs (Fig. 3).

For free swelling (FS) constructs maintained in normoxic conditions, low levels of GAG accumulation was observed in the construct annulus (0.23±0.06 %W.W.), significantly less than in the corresponding core (0.48±0.1 %W.W.) (Fig. 3). This inhomogeneous tissue distribution

was also observed in histological sections stained for alcian blue (Fig. 4). GAG accumulation in the annulus of constructs cultured in low oxygen conditions (0.75±0.06 %W.W.) was lower than in the core (1.46±0.11 %W.W), but still higher than the core of normoxic constructs. Smaller relative differences in collagen accumulation were observed between the core and annular regions of the constructs. Minimal type II collagen staining was evident in the peripheral regions of constructs cultured in normoxic conditions. Normoxic constructs had less intense staining for type II collagen than low oxygen constructs, but slightly more intense staining for type I collagen (Fig. 4).

The differences observed between the core and annular regions of FS constructs were also seen in the loaded constructs. Under normoxic conditions, neither dynamic compression applied from day 0 (CDC), nor delayed until day 21 (DDC), significantly affected GAG accumulation compared to the FS controls. Similarly, in the annulus of constructs cultured in a low oxygen environment, there were no differences between the loading groups. In contrast, within the core regions, both CDC (1.26±0.12 %W.W.) and DDC (1.09±0.19 %W.W.) had lower GAG accumulation compared to FS controls. The collagen/DNA content was also significantly higher in the core of low oxygen FS constructs (47±6.9 μg/μg) compared to CDC (37.1±4.7 μg/μg) and DDC (26.4±5.8 μg/μg).

Construct mechanical properties were higher for all TGF-β3 supplemented groups by day 42 compared to day 0 levels (Fig. 5). A similar trend of increasing mechanical properties with time in culture was not observed in TGF-β3 free groups. In constructs with TGF-β3 supplementation there were significant differences in the mechanical properties between normal and low oxygen culture conditions, with superior mechanical properties observed in low oxygen conditions. There were no differences between the equilibrium and dynamic moduli of normoxic

constructs in the FS, CDC and DDC groups. In contrast, loading in low oxygen culture conditions reduced the dynamic modulus of the tissue by day 42 (FS: 175±13 kPa, CDC: 156±2 kPa, DDC: 134±5 kPa).

4. Discussion:

There are many factors that may regulate endochondral ossification during bone fracture healing. In such a complex environment, it is difficult to determine the regulatory role of individual factors. The objective of this study was to utilize a well developed *in vitro* model of BM MSC chondrogenesis to investigate the role of dynamic compression and oxygen tension, synergistically and in isolation, on the process of bone marrow MSC chondrogenesis. Both of these factors have been hypothesized to regulate chondrogenic differentiation within the callus of a bone fracture, however determining the exact role played by mechanical factors is complicated by the fact that mechanics can also regulate angiogenesis and hence oxygen tension *in vivo*. In the context of MSCs embedded in agarose hydrogels and subjected to continuous low oxygen tension compared with only one hour per day of dynamic compression, this study demonstrates that low oxygen tension is a more potent chondrogenic stimulus than the mechanical signals to which the MSCs are exposed to within the developing agarose constructs. Extrapolating from this finding, this study provides indirect support for the hypothesis that the mechanical environment within a fracture callus exerts its influence on endochondral ossification by inhibiting neovascularization (Ferguson et al., 1999) rather than by any mechanical signals that are directly transduced to the cell. However, as numerous other studies have demonstrated that mechanical signals can promote chondrogenesis of MSCs (Kelly and Jacobs, 2010), this finding may be unique to the experimental set-up utilized in this study. These conditions will be

discussed in greater detail in the proceeding discussion. Nevertheless, the results of this study warrant further investigation of this hypothesis.

Determining the exact mechanism through which biophysical stimuli regulate or influence MSC differentiation has so far proven elusive in the field of mechanobiology. Previous studies have demonstrated that either dynamic compression (Angele et al., 2004; Huang et al, 2004; Campbell et al., 2006; Terraciano et al., 2007; Mauck et al., 2007 ; Thorpe et al., 2008; Kisiday et al., 2009 ; Li et al., 2009) or oxygen tension (Lennon et al., 2001; Schere et al., 2004; Robbins et al., 2005 ; Krimmer et al., 2009; Kanichai et al., 2008) can regulate chondrogenesis of MSCs. Huang et al. (2004) observed that dynamic compression in the absence of chondrogenic growth factors increases the expression of chondrogenic markers as effectively as TGF-β supplementation. It was further suggested that this upregulation in response to mechanical loading was achieved by inducing the synthesis of endogenous TGF-β1, thereby stimulating MSC differentiation. Other studies have confirmed that dynamic compression increases sGAG synthesis and accumulation (Mauck et al., 2007; Kisiday et al 2009), however reported levels of accumulation are significantly lower than that observed in growth factor stimulated groups. In this study, neither dynamic compression nor a low oxygen tension, or a combination or both stimuli, produced robust chondrogenesis of MSCs in the absence of TGF-β3. Differences in species, loading conditions and MSC isolation and expansion protocols may partially explain these differences. The response of MSCs to dynamic compression in the presence of members of the TGF-β superfamily are generally more complex, with anabolic and catabolic responses reported depending on the stage of chondrogenesis that loading is initiated (Lee and Bader, 1997; Mouw et al., 2007; Huang et al., 2010; Thorpe et al., 2010). Under normoxic conditions, we found that dynamic compression did not significantly influence GAG accumulation. As one of

the mechanisms through which dynamic compression exerts its effects is by upregulating TGF-β3 production (Li et al., 2009), the impact of loading may be masked in this study by continuous supplementation of TGF-β3 to the culture media.

GAG accumulation at least doubled in constructs maintained in low oxygen conditions for all experimental arms (Fig. 2), with increases in collagen accumulation also observed. This finding generally agrees with previous studies which demonstrate that low oxygen conditions enhance chondrogenesis of MSCs (Lennon et al., 2001; Kanichai et al., 2008; Krinner et al., 2009; Buckley et al., In Review). Normoxic culture of MSCs produces an inhomogeneous distribution of GAG (see Fig. 4), with greater core GAG accumulation, which may be reflective of the oxygen tension distribution that is developed within the construct (Lewis et al., 2005; Zhou et al., 2008). The side and top surface are readily supplied with oxygen from the culture media, but due to cellular oxygen consumption the microenvironment within the central region will be different from the peripheral domain, experiencing a lower oxygen tension (Guaccio et al., 2008). This low oxygen tension may be the reason for the greater GAG accumulation in the construct cores. The higher core GAG accumulation levels in constructs maintained in low oxygen conditions further suggests that oxygen tensions lower than 5% may be more chondrogenic for BM derived MSCs. A similar finding was observed in both the CDC and DDC loading groups, which further suggests that hypoxia is a more potent chondrogenic stimulus than the mechanical signals generated by dynamic compression.

No synergetic effects were observed by subjecting MSCs to both dynamic compression and low oxygen conditions. Under low oxygen conditions, both CDC and DDC reduced GAG accumulation in the core of constructs compared to free swelling controls. Previous studies have investigated the influence of mechanical loading and oxygen tension on tissue-engineered

chondrocyte-scaffold constructs (Wernike et al., 2007). They demonstrated that low oxygen tension also promoted GAG accumulation and chondrogenic gene expression, and a combination of low oxygen and mechanical loading was shown to reduce collagen I gene expression. In this study, low oxygen culture produced much greater staining for type II collagen and slightly less staining for type I collagen than normoxia, as determined by immunohistochemistry.

Neither low oxygen tension, nor dynamic compression, alone or in combination, induced significant levels of cartilage matrix production in the absence of TGF-β3 supplementation. This may be partially due to the utilization of agarose as a biomaterial to encapsulate bone marrow derived MSCs. One advantage of employing agarose is that it provides a homogenous aqueous environment for the cells, thereby facilitating relatively simple computation of the spatial patterns of biophysical stimuli within the hydrogel using finite element modeling. However, the cells themselves do not attach directly to agarose, which may affect signal transduction pathways (in the early stages of chondrogenesis) that depend on binding of membrane proteins to the surrounding pericellular environment. The stiffness of the substrate itself is also known to regulate MSC differentiation (Winer et al., 2009). MSCs encapsulated in fibrin gel, where greater cell-matrix interactions are known to occur compared to agarose, have been shown generate endogenous TGF-β in response to loading (Li et al., 2009). The inability of MSCs to actively bind to agarose and generate significant cytoskeleton tension may be one reason why they do not respond robustly to either low oxygen or mechanical signals alone. Therefore, we cannot rule out the possibility that substrate materials that better mimic the fracture callus, and allow cell attachment, may initiate chondrogenesis by directly promoting autocrine TGF-β3 signaling in response to mechanical loading.

This model of MSC chondrogenesis, while considering the low oxygen tension and aspects of the mechanical environment present within a fracture callus, is also lacking many factors that are known to be present within the regenerating bone callus. Principally, besides the inclusion of TGF-β3, the model does not include many of the cytokines and growth factors known to be present within the regenerating tissue, as well as the inflammatory cells that at least partially regulate the production of these factors. It is unknown how these factors may interact with mechanical signals and the oxygen tension to regulate chondrogenesis of BM derived MSCs. Furthermore, while dynamic compression of cell encapsulated agarose hydrogels mimics a certain aspect of the loading to which MSCs within a fracture callus would experience, the levels of hydrostatic pressure developed in this *in vitro* model have been estimated (through computational models) to be orders of magnitude lower than those created *in vivo*. Previous studies have demonstrated that high levels of hydrostatic pressure can enhance chondrogenesis of MSCs (Angele et al., 2003), with other studies reporting no effect (Zeiter et al., 2009), however to the authors knowledge no study had considered the combined effect of low oxygen and high levels of hydrostatic pressure on chondrogenesis of MSCs. Future studies will investigate how these factors interact to regulate the MSC differentiation pathway.

This *in vitro* model is therefore lacking many key aspects of the fracture callus environment. However, we can none the less make some important conclusions concerning the role of the mechanical environment in regulating chondrogenesis of MSCs. Specifically, this study provides support for the hypothesis that low oxygen tension is a more potent chondrogenic stimulus for BM derived MSCs than dynamic compression. Such hypothesis can be further tested using computational mechano-regulation models that consider how loading can inhibit angiogenesis (Checa and Prendergast, 2009), and investigating if regulation of MSC

differentiation based purely on the levels of oxygen within a callus are sufficient to predict the spatial and temporal patterns of tissue differentiation during fracture healing. Only by understanding the normal regenerative capabilities of the body can we begin to design and optimize the next generation of treatment options for complex fractures and other bone defects.

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Conflict of Interest Statement:

The authors do not have any conflicts of interest to disclose.

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Legends:

Figure 1: Schematic of experimental design. N=5 samples for each group at each time point. Groups were separated into normal (21%) and low oxygen (5%) incubators, maintained in medium without or with TGF-β3 supplementation and loading consisting of free swelling (FS), continuous dynamic compression (CDC) or delayed dynamic compression (DDC).

Figure 2: Time dependant biochemical properties for normal and low oxygen, loading groups and growth factor supplementation. (A) DNA content (% w/w) (B) GAG content (% w/w) (C) GAG/DNA content (μg/μg) (D) Collagen content (% w/w) (E) Collagen/DNA content(μg/μg). Significance ($p < 0.05$) compared to similar group at: (a) Day 0 (b) Day 21 (c) +TGF-β3 (d) CDC (e) DDC (f) Low oxygen. N=3 samples for each group at each time point.

Figure 3: Day 42 annulus-core biochemical properties for normal and low oxygen and loading groups. (A) DNA content (% w/w) (B) GAG content (% w/w) (C) GAG/DNA content (μg/μg) (D) Collagen content (% w/w) (E) Collagen/DNA content (μg/μg). Significance ($p < 0.05$) compared to similar group at: (a) Core (b) CDC (c) DDC (d) Low oxygen. N=3 samples for each group at each time point.

Figure 4: Histological full cross sections (4x) for normal and low oxygen and dynamically compressed constructs at day 42. Alcian blue staining for sulphated proteoglycan, picro-sirius red for collagen and collagen types II and I immunohistochemistry.

Figure 5: Time dependant mechanical properties for normal and low oxygen, loading groups and growth factor supplementation. (A) Equilibrium modulus (kPa) (B) 1 Hz. dynamic modulus (kPa). Significance ($p < 0.05$) compared to similar group at: (a) Day 0 (b) Day 21 (c) +TGF-β3 (d) CDC (e) DDC (f) Low oxygen. N=3 samples for each group at each time point.

Figures:

Figure 1

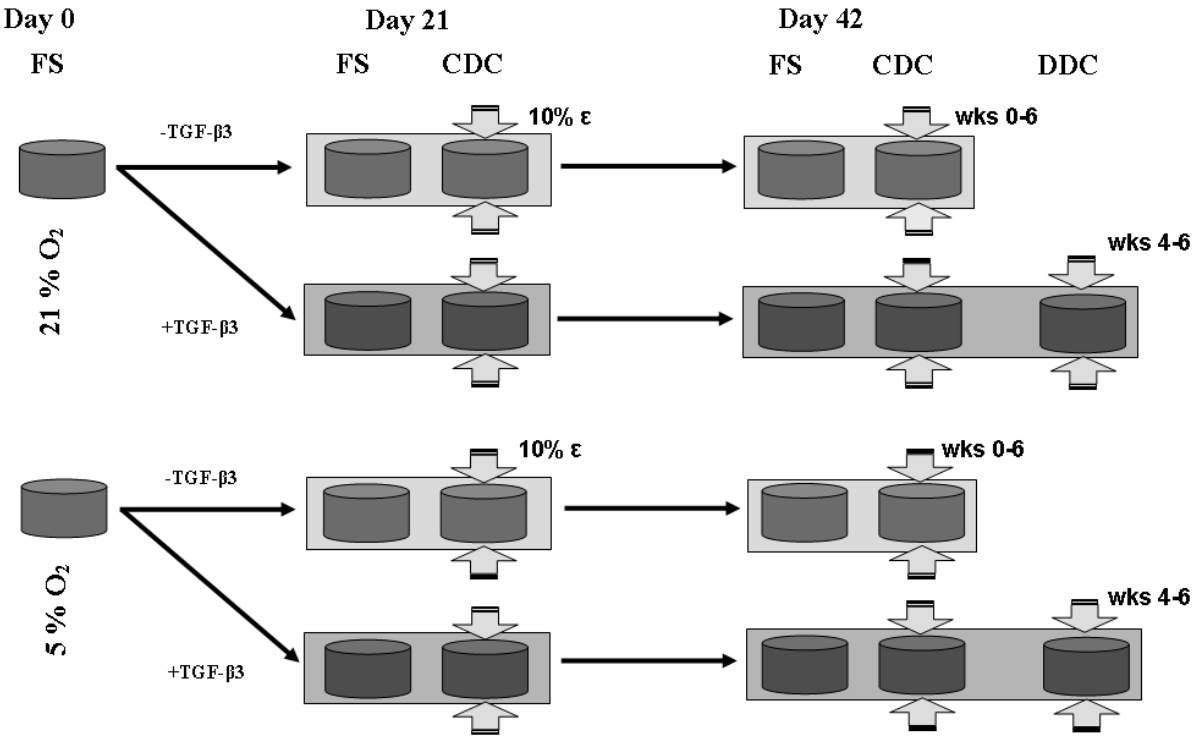


Figure 2

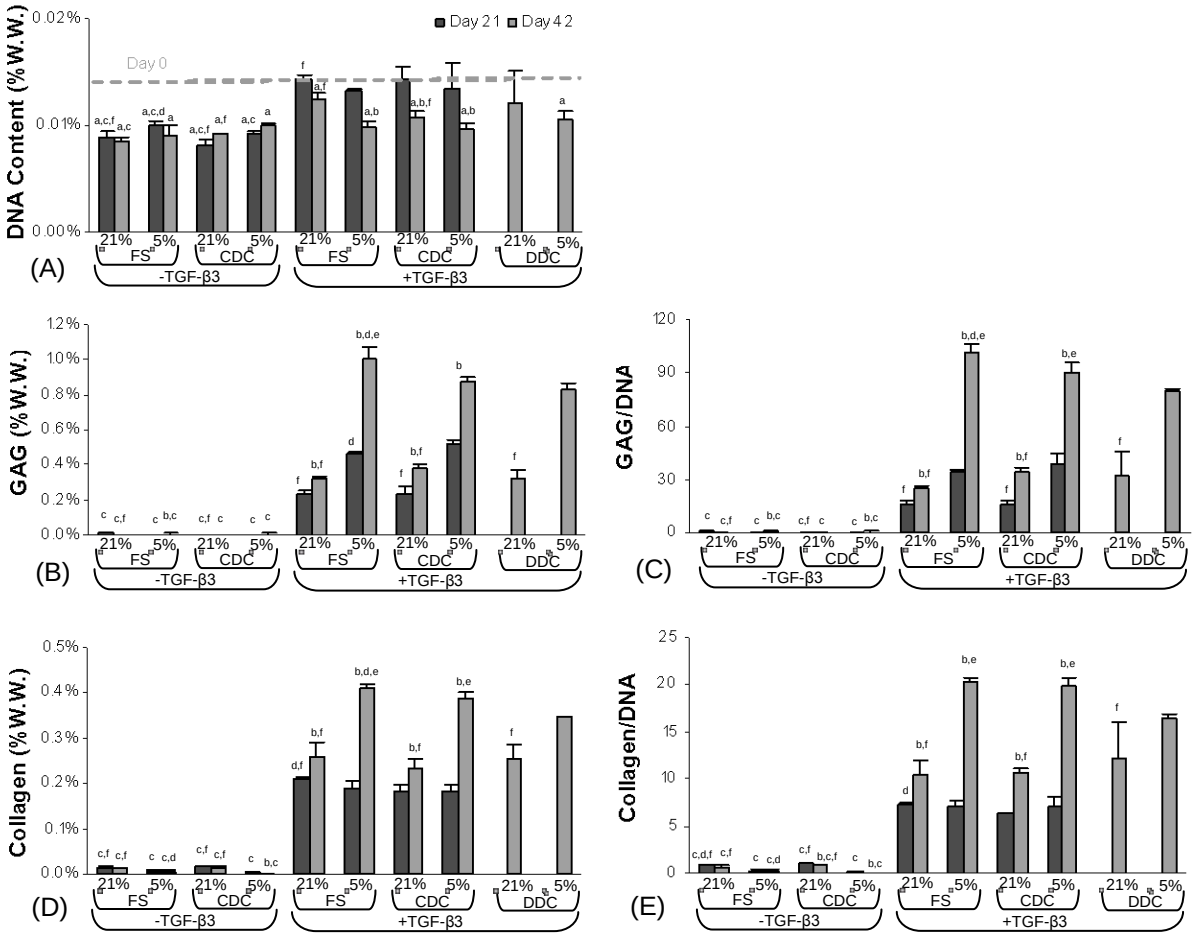

DNA Content (%W.W.)
0.02%
0.01%
0.00%
Day 21
Day 42
Day 0
21% 5%
FS
CDC
DDC
-TGF-β3
+TGF-β3
(A)
GAG (%W.W.)
1.2%
1.0%
0.8%
0.6%
0.4%
0.2%
0.0%
(B)
GAG/DNA
120
90
60
30
0
(C)
Collagen (%W.W.)
0.5%
0.4%
0.3%
0.2%
0.1%
0.0%
(D)
Collagen/DNA
25
20
15
10
5
0
(E)

Figure 3

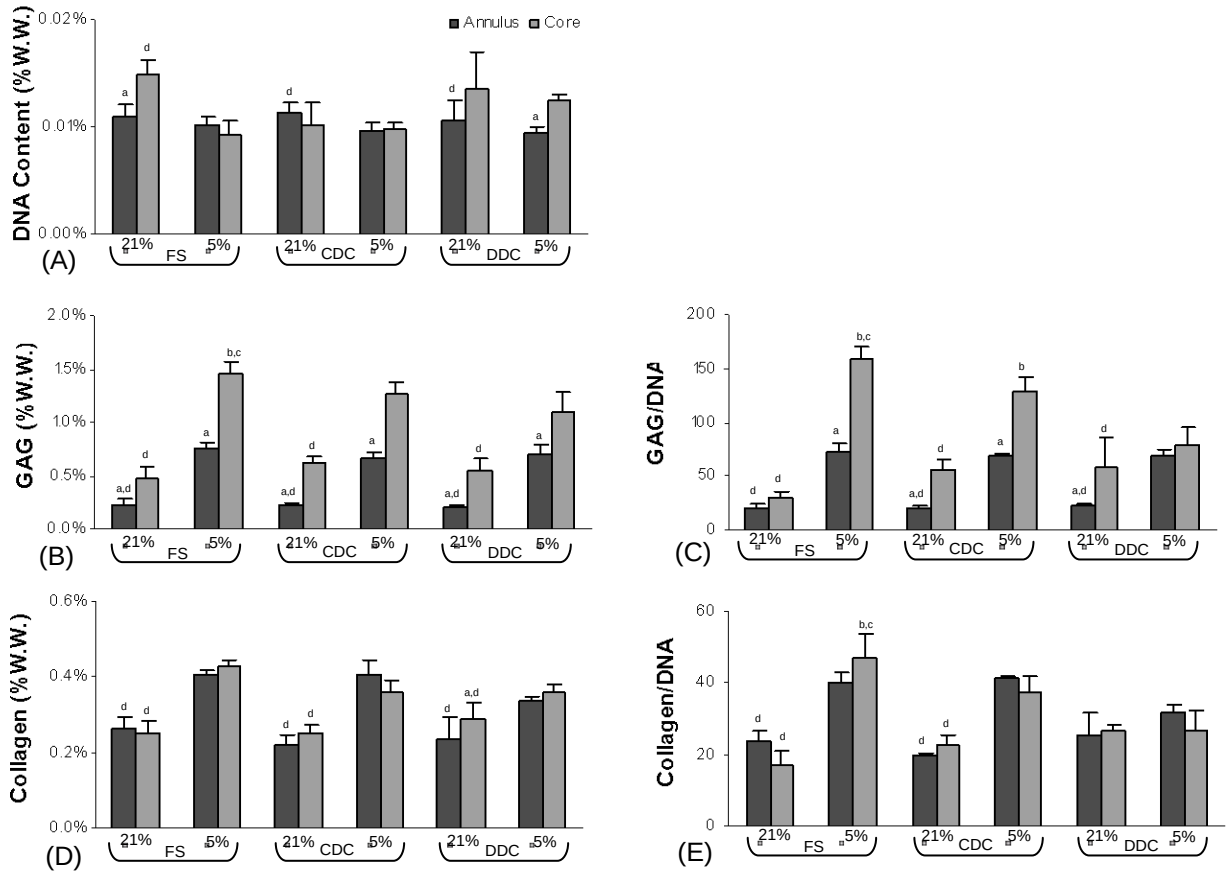

Annulus
Core
DNA Content (%W.W.)
0.02%
0.01%
0.00%
21%
5%
FS
CDC
DDC
(A)
GAG (%W.W.)
2.0%
1.5%
1.0%
0.5%
0.0%
(B)
GAG/DNA
200
150
100
50
0
(C)
Collagen (%W.W.)
0.6%
0.4%
0.2%
0.0%
(D)
Collagen/DNA
60
40
20
0
(E)

Figure 4

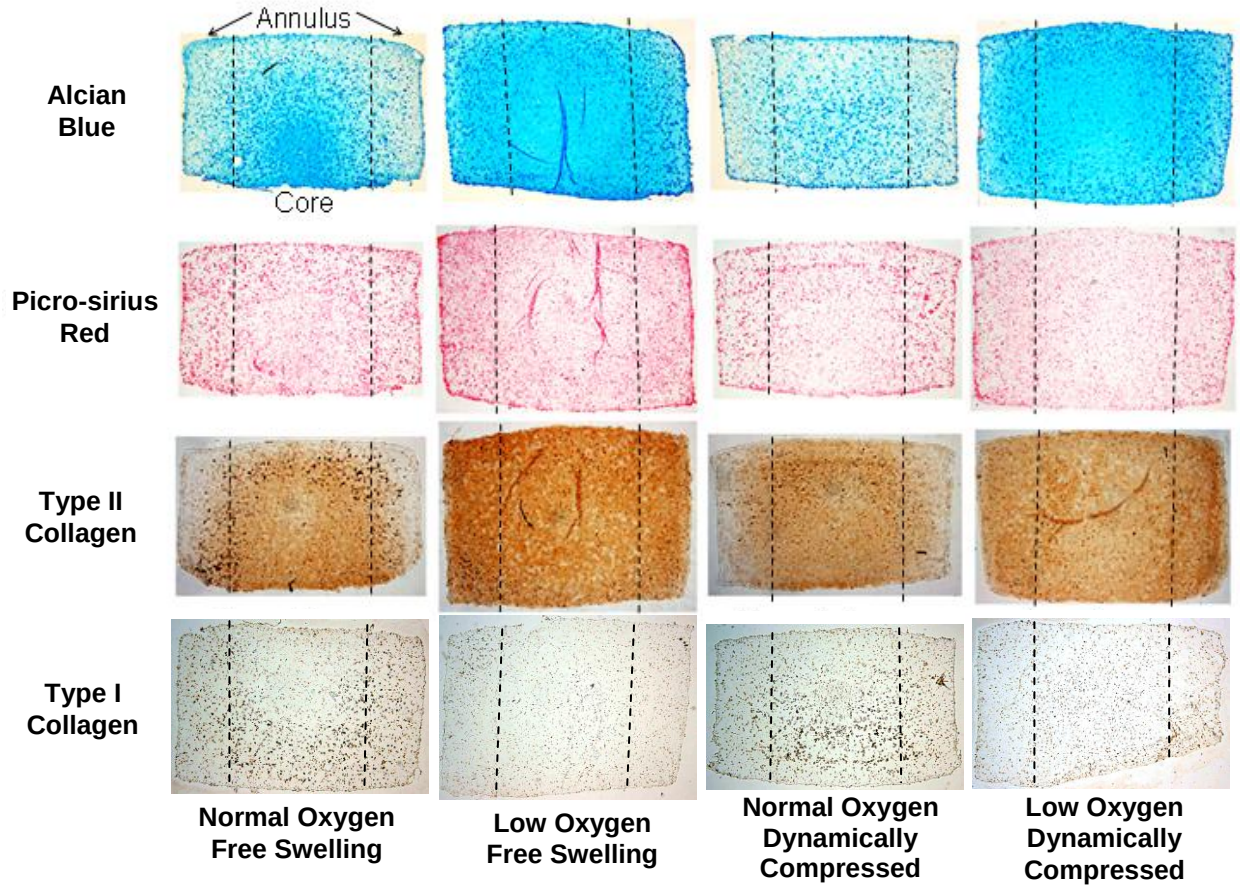
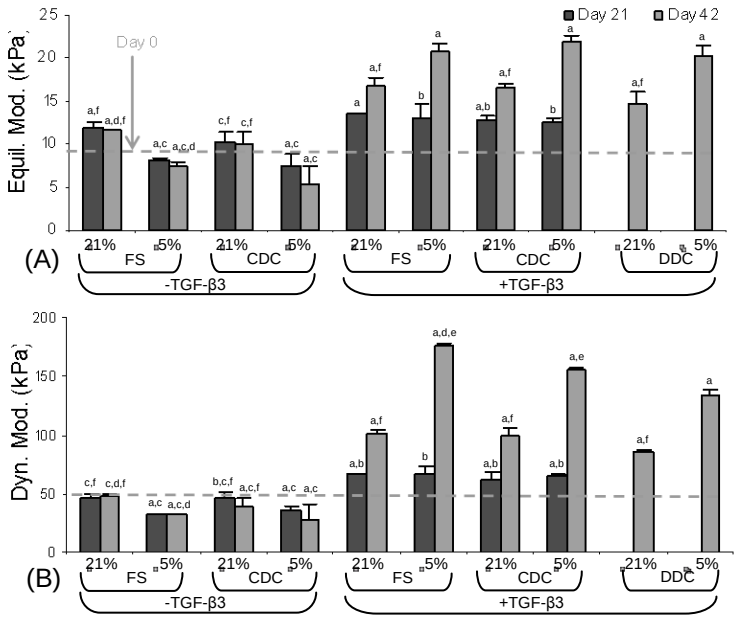

Annulus
Core
Alcian Blue
Picro-sirius Red
Type II Collagen
Type I Collagen
Normal Oxygen Free Swelling
Low Oxygen Free Swelling
Normal Oxygen Dynamically Compressed
Low Oxygen Dynamically Compressed

Figure 5


Day 21
Day 42
Day 0
Equil. Mod. (kPa)
Dyn. Mod. (kPa)
(A)
(B)
21%
5%
FS
CDC
DDC
-TGF-β3
+TGF-β3

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April 6, 2010

Farshid Guilak, Ph.D.
Editor-in-Chief
Journal of Biomechanics

Re: Submission "Low oxygen tension is a more potent regulator of chondrogenic differentiation than dynamic compression"
Authors: Eric G. Meyer, Conor T. Buckley, Stephen D. Thorpe and Daniel J. Kelly

Conflict of Interest Statement:

The authors do not have any conflicts of interest to disclose.

Sincerely,

Eric Meyer

Daniel Kelly