Bone Marrow Stem Cells in Response to Intervertebral Disc-Like Matrix Acidity and Oxygen Concentration - Implications for Cell-Based Regenerative Therapy

Syeda M. Naqvi, M.Sc. [a,b] and Conor T. Buckley, Ph.D. [a,b*]

[a] Trinity Centre for Bioengineering, Trinity Biomedical Sciences Institute, Trinity College Dublin, Ireland.

[b] Department of Mechanical Engineering, School of Engineering, Trinity College Dublin, Ireland.

*Corresponding author: Conor T. Buckley

E-mail address: conor.buckley@tcd.ie

Address: Trinity Centre for Bioengineering

Trinity Biomedical Sciences Institute

Trinity College Dublin

Ireland

Telephone: +353-1-896-2061

Fax: +353-1-679-5554

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Introduction

The harsh microenvironment within the degenerated intervertebral disc (IVD), as characterised by reduced oxygen [1], reduced glucose [2,3] and matrix acidity [4], may limit the success of stem cell-based regeneration of the IVD. Previously, we have reported that for bone marrow stem cells (BMSCs), 5mM glucose and 5% oxygen increased the accumulation of sulphated-glycosaminoglycans (sGAG) and collagen, two important constituents of native nucleus pulposus (NP) tissue, suggesting that IVD-like low glucose and low oxygen is favourable for the biological behaviour of BMSCs [5]. Although this disc environment favours differentiation towards NP-like cells, matrix acidity may hinder survival and matrix synthesis capacity. The pH in a healthy disc ranges from 7.0 to 7.2 [6] and is lower than that of blood (pH 7.4) [7] with the pH in a mildly degenerated disc ranging from 6.7 to 6.9. Importantly, matrix acidity increases further with pH 6.5 being most representative of severely degenerated discs [4].

Matrix acidity has previously been determined to be a crucial factor in stem cell behaviour; an acidic environment decreases proliferation and viability, alters cellular morphology and inhibits anabolic gene expression [8,9]. However, the response of stem cells to varying pH environments remains largely unknown in 3D hydrogels and especially in combination with altered oxygen; numerous studies have investigated the effect of 20% oxygen only [8,10]. Here, we investigate the effect of low (5%) oxygen also which more closely mimics the native disc microenvironment. Taking this into consideration, the overall objective of this study was to investigate the effect of varying pH to simulate typical in vivo environments (pH 7.4: blood, pH 7.1: healthy IVD, pH 6.8: mildly degenerated IVD and pH 6.5: severely degenerated IVD) on the capacity of BMSCs to synthesize NP-like matrix under 20% oxygen (20% O_2) and 5% oxygen (5% O_2) in 3D hydrogels. Importantly, promoting tissue repair with stem cells typically requires the use of high levels of growth factor proteins,

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a task that makes transplantation of stem cells challenging from a regulatory perspective. Hence, the secondary objective of this study was to investigate the effect of varying doses of TGF-β3 (0, 0.1, 1, 10 ng/ml) on the capacity of BMSCs to synthesize NP-like matrix for those maintained under mildly degenerated pH conditions of 6.8.

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Materials and Methods

pH stabilisation and study design

Media with four different pH values were prepared by adding an appropriate amount of hydrochloric acid (HCl) to supplemented low-glucose Dulbecco's modified eagles media (LG-DMEM, 1mg/ml D-Glucose). Briefly, 12 µL/mL of 1M, 2M or 3M HCl was added to supplemented LG-DMEM to ultimately obtain pH 7.1, 6.8 and 6.5 respectively. The pH-adjusted media together with standard supplemented LG-DMEM (control, pH 7.4) was incubated for 18 hours in a humidified incubator (5% CO_2) to allow buffer equilibrium (CO_2-dependent). The desired pH values were obtained after equilibration and were maintained for up to 72 hours, observed both colorimetrically and quantitatively (data not shown).

BMSC isolation and culture

Porcine BMSCs were isolated from the femora of 4-month-old porcine donors (~50kg) within 2 hours of sacrifice and plated at 10×10^6 cells in T-75cm^2 flasks to allow for colony formation. Cultures were maintained in LG DMEM supplemented with 10% foetal bovine serum (FBS), penicillin (100 U/mL)-streptomycin (100 µg/mL) (all GIBCO, Invitrogen, Dublin, Ireland) and amphotericin B (0.25µg/mL, Sigma-Aldrich, Arklow, Ireland). After P0, cells were re-plated at 5×10^3 cells/cm^2 and expanded to passage two (P2) in a humidified atmosphere at 37°C and 5% CO_2.

2D culture under varying pH conditions

Expanded BMSCs were trypsinised, counted using trypan blue staining and seeded onto T25 flasks at a density of $1\text{x}10^4$ cells/cm^2. Cells were maintained in pH (6.5, 6.8, 7.1, 7.4) adjusted chemically defined medium (CDM) consisting of LG-DMEM supplemented with penicillin (100 U/mL)-streptomycin (100 µg/mL), (both from GIBCO, Invitrogen, Dublin,

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Ireland), 0.25 µg/mL amphotericin B, 40 µg/mL L-proline, 1.5 mg/mL bovine serum albumin, 4.7 µg/mL linoleic acid, 1× insulin–transferrin–selenium, 50 µg/mL L-ascorbic acid-2-phosphate and 100 nM dexamethasone in hypoxic (5% O_2) and normoxic oxygen (20% O_2) environments for 7 days. Each flask was maintained in 3mL of CDM with one complete media change performed three days after seeding. Cell proliferation was assessed at day 0 and 7 in terms of DNA content and morphology (crystal violet staining).

Alginate hydrogel encapsulation and culture under varying pH conditions and varying doses of TGF-β3

Expanded BMSCs were trypsinised, counted using trypan blue staining and encapsulated in 1.5% alginate (Pronova UP LVG; FMC NovaMatrix, Sandvika, Norway) at a density of 4×10^6 cells/mL. The alginate/cell suspension was passed through a 12G needle and crosslinked in 102mM calcium chloride ($CaCl_2$) for 20 minutes to produce beads (Ø 5mm).

Beads were maintained in pH (6.5, 6.8, 7.1, 7.4) adjusted chemically defined medium (CDM) consisting of LG-DMEM supplemented with penicillin (100 U/mL)-streptomycin (100 µg/mL), (both from GIBCO, Invitrogen, Dublin, Ireland), 0.25 µg/mL amphotericin B, 40 µg/mL L-proline, 1.5 mg/mL bovine serum albumin, 4.7 µg/mL linoleic acid, 1× insulin–transferrin–selenium, 50 µg/mL L-ascorbic acid-2-phosphate, 100 nM dexamethasone and TGF-β3 (10ng/ml) (all Sigma-Aldrich, Arklow, Ireland) in hypoxic (5% O_2) and normoxic (20% O_2) oxygen environments for 21 days. In parallel, for beads maintained in mildly degenerated IVD-like pH medium (6.8) conditions, varying doses of TGF-β3 supplementation were investigated; 0 ng/mL, 0.1 ng/mL, 1 ng/mL or 10 ng/mL TGF-β3 (PeproTech, UK). Beads were cultured in 24-well plates with one bead per well. Each bead was maintained in 2mL of supplemented CDM with complete media changes performed

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twice weekly for the total culture duration of 21 days. Beads were assessed at days 0 and 21 in terms of cell viability, biochemical content and histologically.

Assessment of Cell viability

Cell viability was assessed using a LIVE/DEAD® Viability/Cytotoxicity Assay Kit (Invitrogen, Bio-science, Ireland). Briefly, constructs were incubated in live/dead solution containing 2μM calcein AM (intact cell membrane) and 4μM ethidium homodimer-1 (disrupted cell membrane; both from Cambridge Bioscience, Cambridge, UK). Sections were imaged with an Olympus FV-1000 Point-Scanning Confocal Microscope at 515 and 615 nm channels and analysed using FV10-ASW 2.0 Viewer software.

Quantitative biochemical analysis

Samples were digested with 3.88 U/mL papain in 0.1 M sodium acetate, 5 mM L-cysteine HCl and 0.05 M EDTA (pH 6.0) (all from Sigma-Aldrich) at 60°C under constant rotation for 18 hours followed by the addition of 1M sodium citrate solution for 1hr under same the conditions. DNA content was determined using the Hoescht 33258 dye-based assay (DNA QF Kit,Sigma- Aldrich, Ireland). Proteoglycan 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 hours in 12M HCl and assayed using a chloramine-T assay [11] with a hydroxyproline:collagen ratio of 1:7.69 [12].

Histology and immunohistochemistry

At each time point samples were fixed in 4% paraformaldehyde overnight, dehydrated in a graded series of ethanols, embedded in paraffin wax, sectioned at 8μm and affixed to

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microscope slides. Sections were stained with aldehyde fuschin/alcian blue to assess sGAG content and picro-sirius red to assess collagen distribution (all Sigma-Aldrich). Collagen I and II were evaluated using a standard immunohistochemical technique [5,13].

Statistical analysis

Statistical analyses were performed using GraphPad Prism (version 5) software with 3-4 samples analysed for each experimental group. Two-way ANOVA was used for analysis of variance with Bonferroni's post-tests to compare between groups. Graphical results are displayed as mean ± standard deviation. Significance was accepted at a level of $p < 0.05$. The entire experiment was replicated with cells from the second donor, which confirmed the findings presented in this manuscript.

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Results

2D proliferation study results demonstrate that for pH 6.5 at Day 7, DNA content decreased from Day 0 levels for both oxygen concentrations (Supplementary Fig. 1A). Results from DNA analysis were confirmed by crystal violet staining (Supplementary Fig. 1B); a decreased number of adherent cells were observed under pH 6.5. For all other pH values, the DNA content remained at Day 0 levels by Day 7 with the exception of pH 7.4 under normoxic conditions (Supplementary Fig. 1A).

For both oxygen concentrations, constructs decreased in DNA content from day 0 levels for pH 6.5 (Fig. 1A). Results from DNA analysis were confirmed through confocal imaging of live/dead cells (Fig. 1B); an increased number of dead cells (red) were observed in pH 6.5. For pH 6.8, constructs in 20% oxygen illustrated an increase in DNA content compared with 5% oxygen. However, for constructs in pH 7.4, the opposite was observed with increased DNA content in 5% oxygen compared to 20% oxygen (Fig. 1A). With regards to live/dead imaging, cell viability was maintained in both oxygen concentrations for pH 6.8, 7.1 and 7.4 (Fig. 1B).

Total sGAG content was significantly higher in 5% oxygen compared with 20% oxygen for pH 7.1 and 7.4. In addition to these differences, there was an observed increase in sGAG content in 5% oxygen compared to 20% oxygen for pH 6.8 when sGAG was normalized to DNA (Fig. 2A). For pH 6.5, there was little to no sGAG accumulation regardless of external oxygen concentration. Interestingly, the differences observed between the different pH values were only evident for those maintained in 5% oxygen with increased sGAG content observed in pH 7.1, representative of healthy IVD–like pH conditions. These differences were not apparent for constructs maintained in 20% oxygen. Histological evaluation confirmed the biochemical findings demonstrating greater sGAG deposition in 5% oxygen compared with 20% oxygen (Fig. 2B); aldehyde fuchsin/alcian blue staining revealed

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greater sGAG accumulation in 5% oxygen for pH 6.8, 7.1 and 7.4 compared with 20% oxygen. Furthermore, for pH 6.5, constructs illustrated no sGAG accumulation as demonstrated by light blue staining indicating residual alginate only.

With regards to collagen content, there was little to no collagen content observed at pH 6.5 (Fig. 2C). For pH 6.8, 20% oxygen displayed significantly greater total collagen content compared with 5% oxygen. However, when normalized to DNA, this difference was not apparent. This may be explained by the differences in DNA content between 20% and 5% oxygen whereby DNA content in 20% oxygen conditions was higher at pH 6.8. For pH 7.1, the increased collagen content in 5% oxygen compared to 20% oxygen is maintained when collagen is normalised to DNA. Histological evaluation confirmed biochemical analysis findings, demonstrating no collagen deposition in pH 6.5 regardless of oxygen concentration and increased collagen deposition in constructs for pH 6.8 or higher (Fig. 2D).

For pH 6.5, immunohistochemistry results revealed limited collagen I and II accumulation regardless of oxygen concentration (Fig. 3). For pH 6.8 or higher, results demonstrated increased collagen II deposition in 5% oxygen and increased collagen I deposition in 20% oxygen. With regards to collagen II, there were no apparent differences in the amount of collagen accumulation in 20% oxygen, although for 5% oxygen there appears to be more collagen II deposited at pH 7.1 within the core. With regards to collagen I, there were no apparent differences in the amount of collagen accumulation in 5% oxygen, although for 20% oxygen there appeared to be more collagen I deposition at pH 6.8.

Constructs maintained in media without TGF-β3 resulted in negligible accumulation of matrix (sGAG and collagen) irrespective of oxygen concentration (Fig. 4). sGAG content increased significantly with TGF-β3 from 0.1ng/ml to 10ng/ml ($P<0.05$) (Fig 4A). Histological evaluation confirmed biochemical findings with negligible deposition of sGAG in the absence of TGF-β3 and increased deposition with increasing concentration of TGF-β3.

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With regards to collagen content, this dosage effect diminished for those maintained in 20% oxygen with similar collagen content observed in 0.1ng/ml TGF-β3 or higher. However, for those maintained in 5% oxygen, the dose effect was present although less pronounced than that for sGAG content with increased collagen content concomitant with increased TGF-β3 from 0.1ng/ml to 10ng/ml with a significant increase from 0.1ng/ml to 1ng/ml ($P<0.05$) (Fig 4B).

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Discussion

To identify the optimal timing for intervention that will offer the greatest regenerative potential, the effect of decreasing pH on BMSC activity was investigated correlating with the degenerative stage of the IVD. The pH values were chosen to simulate values in blood vessels surrounding the IVD (7.4) and values reported to exist within the nucleus pulposus region of a healthy IVD (7.1), a mildly degenerated IVD (6.8) and a severely degenerated IVD (6.5).

In cell-based interventions, the ability of cells to survive and synthesize disc-like matrix in vivo after transplantation into the central NP region is crucial. In this regard, the stage of degeneration is critical where the results from this study suggest the limited regenerative potential of BMSCs below pH 6.8. At or above this threshold, we demonstrate that BMSCs exhibit sustained cell viability with increased sGAG and collagen accumulation. Below this threshold, at pH 6.5, cell survival was diminished concomitant with decreased DNA content in both 2D and 3D culture. Interestingly, for pH 7.4 an observed contrasting result was found between both 2D and 3D culture conditions with increased proliferation in normoxia compared to hypoxia for 2D and the opposite for 3D culture. It has previously been reported that hypoxia decreases cell proliferation in 2D culture for adipose derived stem cells [14]. In 3D culture, encapsulated cells respond to proliferative signals through interactions mediated by the surrounding matrix [15]. This may in part explain the observed results of increased proliferation (Figure 1A) in 3D culture due to the concomitant increase in matrix production under hypoxic conditions (Figure 2A). Importantly, pH 6.5 was found to be detrimental to matrix synthesis. This correlates with a study by Bibby et al, who found a decrease in NP cell viability when decreasing the pH to 6.7 and a more obvious decrease for pH 6.2 [3]. In another study, Han et al examined the viability and matrix metabolism of nucleus pulposus mesenchymal stem cells (NPMSCs) and adipose tissue-derived

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mesenchymal stem cells (ADMSCs) under similar acidic conditions in vitro and observed that acidic pH inhibited cell viability and down regulated the expression of aggrecan, collagen I and II for both cell types, thus demonstrating that an acidic environment is a major challenge for IVD regeneration [10]. This has key implications in the development of stem cell based therapeutic strategies and a degeneration-dependent stratification of intervention is proposed. Through measurement of IVD pH, suitable candidates can be identified for successful outcomes where stem cell transplantation may be more effective at an early stage of IVD degeneration when the pH is higher than 6.8.

Although we previously demonstrated that BMSCs can survive and synthesise NP-like matrix in IVD-like low oxygen (5%) and low glucose (5mM) [5], we show in this study that pH is a critical factor in limiting the use of BMSCs in disc repair with pH levels as low as 6.5 being reported in a severely degenerated disc [6,16]. This correlates with a study by Wuertz et al which explored the effects of IVD-like culture conditions on stem cell survival and sGAG/collagen expression. They demonstrated that combining low pH with low glucose is detrimental to the differentiation of stem cells, with decreased cellular proliferation and sGAG/collagen expression suggesting that the beneficial effects of IVD-like low-glucose culture are not sufficient for promotion of stem cell differentiation when other environmental factors are considered such as matrix acidity [9]. Liang et al investigated the viability and expression of aggrecan for ADMSCs under normal and degenerative IVD conditions. They found that IVD-like low glucose conditions increased the expression of aggrecan but IVD-like high acidity inhibited the its expression demonstrating that, although IVD-like low glucose is a positive factor IVD-like low pH is deleterious and affects the biological behaviour of ADMSCs [17].

In addition to simulating IVD-like low glucose conditions, we also explored the influence of both 20% and 5% oxygen and found that low pH conditions (pH 6.5 and 6.8)

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like those found in the degenerating disc were detrimental in both scenarios. However, our data shows that 5% oxygen enhanced sGAG content even at the lower pH value of 6.8. To the best of our knowledge, this is the first study to demonstrate the beneficial effect of 5% oxygen with varying pH conditions on the capacity of stem cells to synthesize matrix. Any differences observed between the different pH values were only evident for those maintained in 5% oxygen with increased sGAG content concomitant with healthier pH conditions. These differences were not apparent for those maintained in 20% oxygen.

Chondrogenic differentiation of MSCs has been shown in the presence of members of the transforming growth factor (TGF)-β superfamily [18-25]. The mechanism by which it promotes MSC differentiation consists of many pathways including the TGF-β, extracellular-signal regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) signalling pathways [26-29]. TGF-β3 is one isoform of the TGF-β super family of signalling molecules. Indeed, we have previously studied a combination of TGF-β3 and BMSCs encapsulated in an injectable hydrogel [30] and found that it stimulates proteoglycan production and increases collagen synthesis. This observation of TGF- β enhancing differentiation of MSCs to NP-like cells has also been reported in other studies [31,32]. Furthermore, other growth factors of the TGF-β superfamily such as BMP-7 have also been investigated and shown to enhance chondrogenic differentiation of MSCs, in combination with TGF-β. For example, Shen et al. demonstrate that BMP-7, when supplemented in chondrogenic differentiation medium containing TGF-β3, enhanced the mRNA and/or protein expression of chondrogenic markers COL2A1, COL9A1, COL10A1, and SOX9, and increased extracellular matrix proteoglycan deposition [33]. Importantly, in this study we demonstrate that despite the fact that BMSCs are multipotent, exogenous growth factor supplementation is required for matrix synthesis in a simulated degenerative IVD environment with 10 ng/mL being the most effective dose, consistent with previous findings [34,35]. This has major implications for stem cell based therapy with regards

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to ECM synthesis capacity and would require the concurrent delivery of growth factor with the regenerative cell population, with the challenge being the relatively short half-life of growth factors which may require serial injections within the clinical setting. Furthermore, regulatory approval of growth factors remains a critical barrier. Of note however, is that an initial dosage may be sufficient to initiate differentiation. For example, Buxton et al showed that an initial exposure to TGF-β followed by its withdrawal resulted in total collagen deposition equivalent to controls (continuous TGF-β exposure for at least 3 weeks) [36]. Furthermore, proteoglycan content per construct was higher at 6 weeks after removal of TGF-β. Also, pre-treatment with TGF-β could prevent differentiated MSCs encapsulated in alginate beads from transdifferentiating into osteoblasts [37]. In light of this, the use of preconditioned or primed BMSCs for transplantation may be an alternative to the concurrent delivery of growth factors combined with cells. BMSCs, which are not committed or adapted to the unique microenvironmental conditions of the degenerating disc, may in fact be unsuited to the hostile environment of the diseased IVD. As such long term survival and integration within the disc may require pre-differentiation of these cells during the in vitro culture period towards a phenotype more representative of native IVD cells. This correlates with other studies, that unconditioned stem cells have a diminished probability of survival in the "harsh" conditions, i.e. low pH, low oxygen and low glucose concentration of the IVD [8,9,38,39]. On the other hand, in a similar culture system, Ichimura et al found that the rate of proteoglycan synthesis in rat NP cells was similar between pH 6.6 and 7.6 [7]. In this regard, compared with NP cells, which are well suited to the harsh IVD microenvironment, acidity change appears to be more detrimental to BMSCs. These differences may be attributed to disc cells possessing effective buffering and acid equivalent transport pathways to regulate intracellular pH [40].

In conclusion, translation into a multimodal protocol requires the survival of injected

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stem cells and their ability to function normally amidst the harsh microenvironment. This study demonstrates the critical implication of degeneration stage and suggests stratified targeting to identify suitable candidates through measurement of the local pH thereby maximising the efficacy for IVD cellular regenerative interventions.

References

1. Grunhagen T, Wilde G, Soukane DM, et al. Nutrient supply and intervertebral disc metabolism. *J Bone Joint Surg Am* 2006;88 Suppl 2:30-5.
2. Bibby SR, Urban JP. Effect of nutrient deprivation on the viability of intervertebral disc cells. *Eur Spine J* 2004;13:695-701.
3. Bibby SR, Jones DA, Ripley RM, et al. Metabolism of the intervertebral disc: effects of low levels of oxygen, glucose, and pH on rates of energy metabolism of bovine nucleus pulposus cells. *Spine (Phila Pa 1976)* 2005;30:487-96.
4. Urban JP. The role of the physicochemical environment in determining disc cell behaviour. *Biochem Soc Trans* 2002;30:858-64.
5. Naqvi SM, Buckley CT. Extracellular matrix production by nucleus pulposus and bone marrow stem cells in response to altered oxygen and glucose microenvironments. *Journal of anatomy* 2015:doi: 10.1111/joa.12305.
6. Kitano T, Zerwekh JE, Usui Y, et al. Biochemical changes associated with the symptomatic human intervertebral disk. *Clin Orthop Relat Res* 1993:372-7.
7. Ichimura K, Tsuji H, Matsui H, et al. Cell culture of the intervertebral disc of rats: factors influencing culture, proteoglycan, collagen, and deoxyribonucleic acid synthesis. *J Spinal Disord* 1991;4:428-36.
8. Wuertz K, Godburn K, Iatridis JC. MSC response to pH levels found in degenerating intervertebral discs. *Biochemical and biophysical research communications* 2009;379:824-9.
9. Wuertz K, Godburn K, Neidlinger-Wilke C, et al. Behavior of mesenchymal stem cells in the chemical microenvironment of the intervertebral disc. *Spine (Phila Pa 1976)* 2008;33:1843-9.
10. Han B, Wang HC, Li H, et al. Nucleus pulposus mesenchymal stem cells in acidic conditions mimicking degenerative intervertebral discs give better performance than adipose tissue-derived mesenchymal stem cells. *Cells Tissues Organs* 2014;199:342-52.
11. Kafienah W, Sims TJ. Biochemical methods for the analysis of tissue-engineered cartilage. *Methods Mol Biol* 2004;238:217-30.
12. Ignat'eva NY, Danilov N, Averkiev S, et al. Determination of hydroxyproline in tissues and the evaluation of the collagen content of the tissues. *Journal of Analytical Chemistry* 2007;62:51-7.
13. Naqvi SM, Buckley CT. Differential response of encapsulated nucleus pulposus and bone marrow stem cells in isolation and coculture in alginate and chitosan hydrogels. *Tissue Eng Part A* 2015;21:288-99.
14. Wang DW, Fermor B, Gimble JM, et al. Influence of oxygen on the proliferation and metabolism of adipose derived adult stem cells. *Journal of cellular physiology* 2005;204:184-91.

Accepted Manuscript

15. Harisi R, Dudas J, Timar F, et al. Invasive growth and topoisomerase-switch induced by tumorous extracellular matrix in osteosarcoma cell culture. *Cell biology international* 2005;29:959-67.
16. Diamant B, Karlsson J, Nachemson A. Correlation between lactate levels and pH in discs of patients with lumbar rhizopathies. *Experientia* 1968;24:1195-6.
17. Liang C, Li H, Tao Y, et al. Responses of human adipose-derived mesenchymal stem cells to chemical microenvironment of the intervertebral disc. *J Transl Med* 2012;10:49.
18. Huang CY, Reuben PM, D'Ippolito G, et al. Chondrogenesis of human bone marrow-derived mesenchymal stem cells in agarose culture. *The anatomical record. Part A, Discoveries in molecular, cellular, and evolutionary biology* 2004;278:428-36.
19. Iwasaki M, Nakata K, Nakahara H, et al. Transforming growth factor-beta 1 stimulates chondrogenesis and inhibits osteogenesis in high density culture of periosteum-derived cells. *Endocrinology* 1993;132:1603-8.
20. Johnstone B, Hering TM, Caplan AI, et al. In vitro chondrogenesis of bone marrow-derived mesenchymal progenitor cells. *Exp Cell Res* 1998;238:265-72.
21. Mackay AM, Beck SC, Murphy JM, et al. Chondrogenic differentiation of cultured human mesenchymal stem cells from marrow. *Tissue engineering* 1998;4:415-28.
22. Nishimura K, Solchaga LA, Caplan AI, et al. Chondroprogenitor cells of synovial tissue. *Arthritis Rheum* 1999;42:2631-7.
23. Palmer GD, Steinert A, Pascher A, et al. Gene-induced chondrogenesis of primary mesenchymal stem cells in vitro. *Molecular therapy : the journal of the American Society of Gene Therapy* 2005;12:219-28.
24. Yoo JU, Barthel TS, Nishimura K, et al. The chondrogenic potential of human bone-marrow-derived mesenchymal progenitor cells. *J Bone Joint Surg Am* 1998;80:1745-57.
25. Zuk PA, Zhu M, Mizuno H, et al. Multilineage cells from human adipose tissue: implications for cell-based therapies. *Tissue engineering* 2001;7:211-28.
26. Risbud MV, Albert TJ, Guttapalli A, et al. Differentiation of mesenchymal stem cells towards a nucleus pulposus-like phenotype in vitro: implications for cell-based transplantation therapy. *Spine (Phila Pa 1976)* 2004;29:2627-32.
27. Massague J, Seoane J, Wotton D. Smad transcription factors. *Genes & development* 2005;19:2783-810.
28. Risbud MV, Di Martino A, Guttapalli A, et al. Toward an optimum system for intervertebral disc organ culture: TGF-beta 3 enhances nucleus pulposus and anulus fibrosus survival and function through modulation of TGF-beta-R expression and ERK signaling. *Spine (Phila Pa 1976)* 2006;31:884-90.
29. Derynck R, Zhang YE. Smad-dependent and Smad-independent pathways in TGF-beta family signalling. *Nature* 2003;425:577-84.
30. Naqvi SM, Buckley CT. Differential Response of Encapsulated Nucleus Pulposus and Bone Marrow Stem Cells in Isolation and Coculture in Alginate and Chitosan Hydrogels. *Tissue Eng Part A* 2015;21:288-99.
31. Luo W, Xiong W, Qiu M, et al. Differentiation of mesenchymal stem cells towards a nucleus pulposus-like phenotype utilizing simulated microgravity In vitro. *J Huazhong Univ Sci Technolog Med Sci* 2011;31:199-203.
32. Morigele M, Shao Z, Zhang Z, et al. TGF-beta1 induces a nucleus pulposus-like phenotype in Notch 1 knockdown rabbit bone marrow mesenchymal stem cells. *Cell biology international* 2013;37:820-5.
33. Shen B, Wei A, Whittaker S, et al. The role of BMP-7 in chondrogenic and osteogenic differentiation of human bone marrow multipotent mesenchymal stromal cells in vitro. *J Cell Biochem* 2010;109:406-16.

Accepted Manuscript

34. Bosnakovski D, Mizuno M, Kim G, et al. Chondrogenic differentiation of bovine bone marrow mesenchymal stem cells in pellet cultural system. *Exp Hematol* 2004;32:502-9.
35. Richter W. Mesenchymal stem cells and cartilage in situ regeneration. *J Intern Med* 2009;266:390-405.
36. Buxton AN, Bahney CS, Yoo JU, et al. Temporal exposure to chondrogenic factors modulates human mesenchymal stem cell chondrogenesis in hydrogels. *Tissue Eng Part A* 2011;17:371-80.
37. Mehlhorn AT, Schmal H, Kaiser S, et al. Mesenchymal stem cells maintain TGF-beta-mediated chondrogenic phenotype in alginate bead culture. *Tissue engineering* 2006;12:1393-403.
38. Zeiter S, Van Der Werf M, Ito K. The fate of bovine bone marrow stromal cells in hydrogels: A comparison to nucleus pulposus cells and articular chondrocytes. *Journal of tissue engineering and regenerative medicine* 2009;3:310-20.
39. Holzwarth C, Vaegler M, Gieseke F, et al. Low physiologic oxygen tensions reduce proliferation and differentiation of human multipotent mesenchymal stromal cells. *BMC Cell Biol* 2010;11:11.
40. Razaq S, Urban JP, Wilkins RJ. Regulation of intracellular pH by bovine intervertebral disc cells. *Cell Physiol Biochem* 2000;10:109-15.

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Figure legends

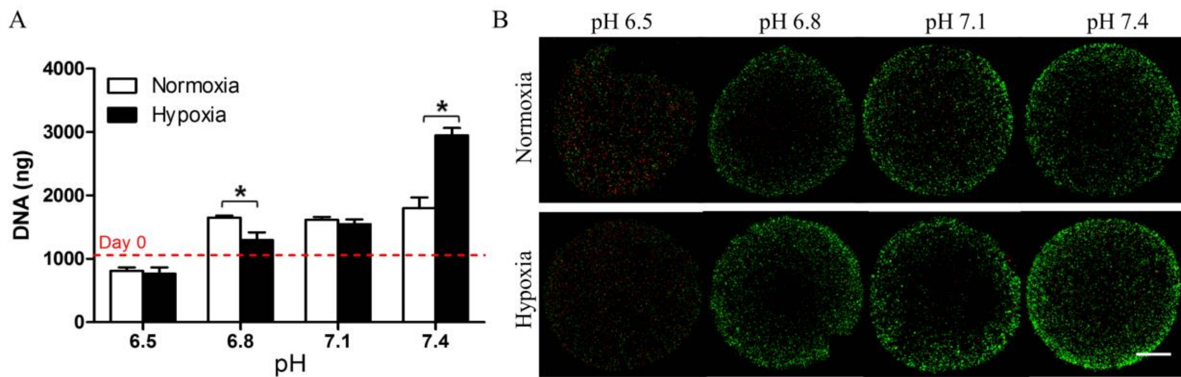


Figure 1: (A) Total DNA content (ng) at day 21 for bone marrow stem cells (BMSCs) maintained under different pH conditions (6.5, 6.8, 7.1 and 7.4) in normoxia (20% O_2) or hypoxia (5% O_2); * denotes significance compared to normoxia for same pH condition ($p<0.05$); dashed line represents Day 0 DNA content **(B)** Cell viability for BMSC constructs at day 21. Scale bar =1mm.

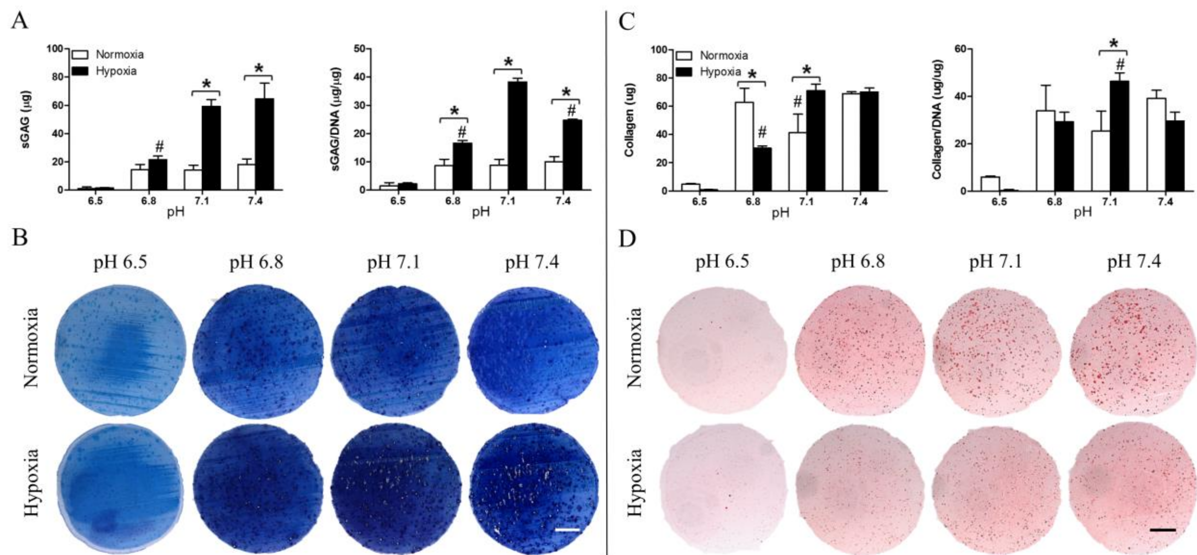


Figure 2: (A) Total sGAG (µg) and sGAG normalized to DNA content (µg/µg) at day 21 for BMSCs maintained under different pH conditions (6.5, 6.8, 7.1 and 7.4) in normoxia (20% O_2) or hypoxia (5% O_2) **(B)** Histological evaluation with aldehyde fuchsin and alcian blue to identify sGAG at day 21; deep blue/purple staining indicates GAG accumulation and light blue staining indicates residual alginate; **(C)** Total collagen (µg) and collagen normalised to

DNA content (µg/µg) at day 21 for BMSCs maintained under different pH conditions (6.5, 6.8, 7.1 and 7.4) in normoxia (20% O_2) or hypoxia (5% O_2) **(D)** Histological evaluation with picrosirius red to identify collagen at day 21; * denotes significance compared to normoxia for same pH condition, # denotes significance compared to all other pH condition for same oxygen concentration. Scale bar = 1mm.

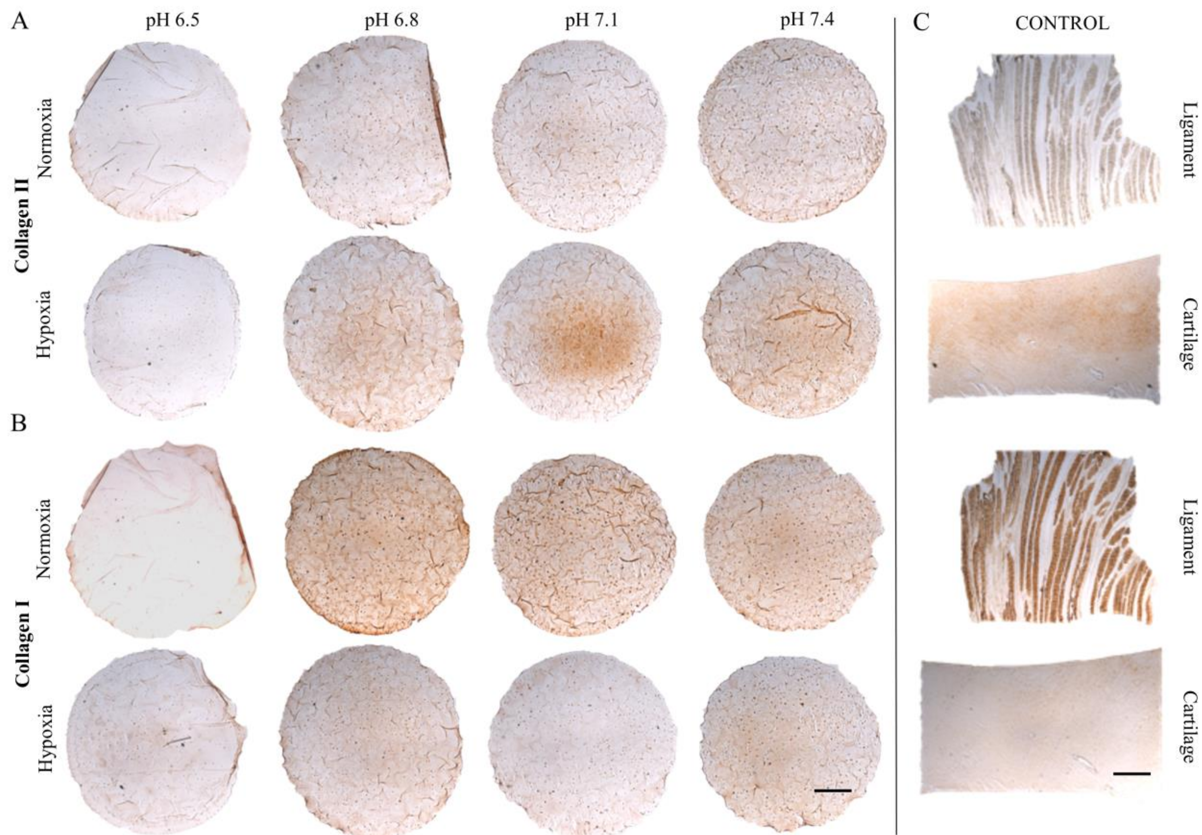


Figure 3: Immunohistochemistry at day 21 for BMSCs maintained under different pH conditions (6.5, 6.8, 7.1 and 7.4) in normoxia (20% O_2) or hypoxia (5% O_2) **(A)** for collagen II; **(B)** for collagen I; **(C)** Ligament and cartilage controls for collagen I and collagen II. Scale bar = 1mm

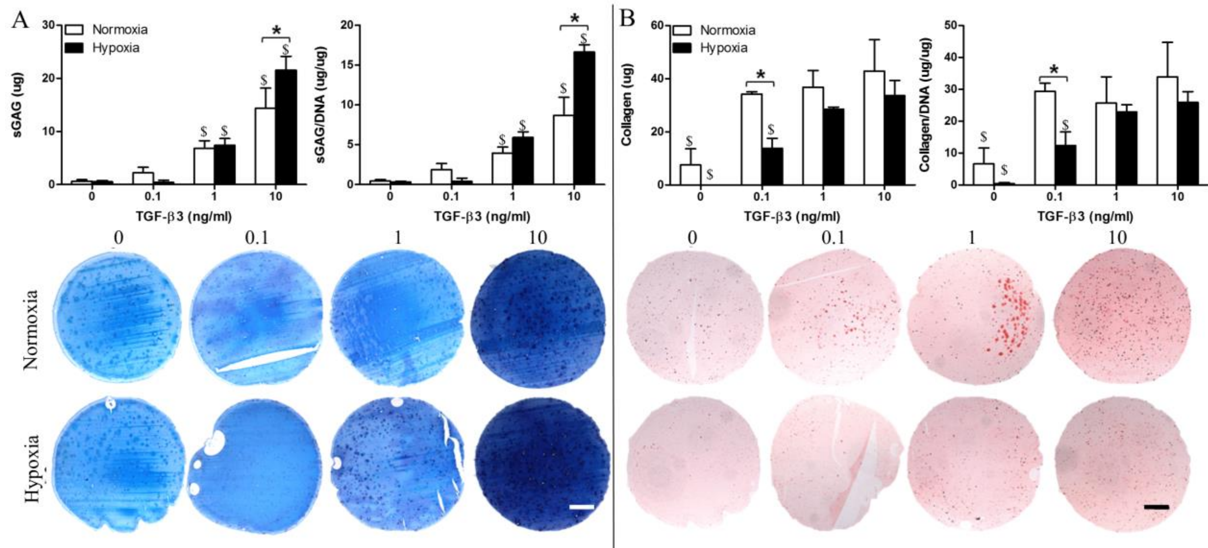
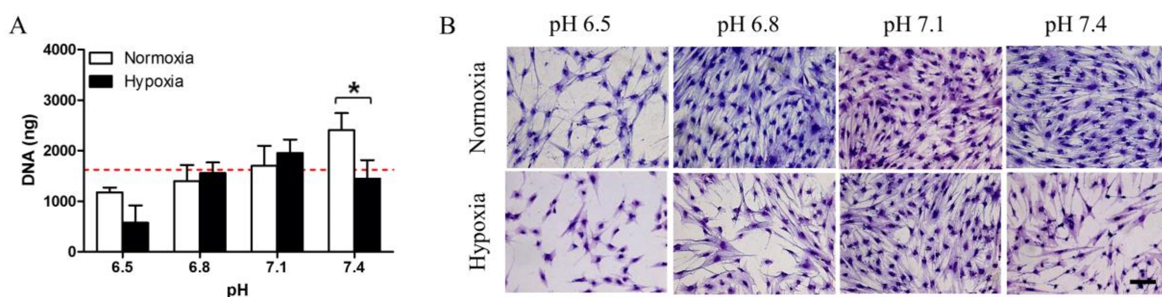


Figure 4: Response to varying doses of TGF-β3 (0, 0.1, 1 and 10 ng/ml) under pH 6.8 conditions at day 21 in normoxia (20% O_2) or hypoxia (5% O_2) **(A)** Total sGAG (µg), sGAG normalized to DNA content (µg/µg) and histological evaluation; aldehyde fuchsin and alcian blue staining: deep blue/purple staining indicates GAG accumulation and light blue staining indicates residual alginate. * denotes significance compared to normoxia for same TGF-β3 concentration, $ denotes significance compared to all other TGF-β3 concentrations for same oxygen concentration. **(B)** Total collagen (µg), collagen normalised to DNA content (µg/µg) and histological evaluation. Scale bar = 1mm.



Supplementary Figure 1: (A) Total DNA content (ng) at day 7 for bone marrow stem cells (BMSCs) maintained under different pH conditions (6.5, 6.8, 7.1 and 7.4) in normoxia (20% O_2) or hypoxia (5% O_2) on 2D culture plastic; * denotes significance compared to normoxia for same pH condition ($p<0.05$); dashed line represents Day 0 DNA content (B) Cell morphology for BMSCs at day 7 using crystal violet staining. Scale bar =100µm.