

PRIMING AS A STRATEGY TO OVERCOME DETRIMENTAL PH EFFECTS ON CELLS FOR INTERVERTEBRAL DISC REGENERATION

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Abstract

Disc disease is characterised by degeneration of the nucleus pulposus (NP), the central gelatinous tissue of the intervertebral disc (IVD). As degeneration progresses, the microenvironment of the IVD becomes more hostile (*i.e.* decrease in oxygen, glucose and pH), providing a significant challenge for regeneration using cell-based therapies. Tissue engineering strategies such as priming cells or micro tissues with growth factors prior to implantation may overcome some of these issues by providing a pre-formed protective niche composed of extracellular matrix. The present study investigated the effect of priming, using transforming growth factor $\beta 3$ (TGF- $\beta 3$), on bone-marrow-derived stem cells (BMSCs) and articular chondrocytes (ACs) cultured at different pH levels (pH 7.1, 6.8 and 6.5) representative of the *in vivo* disc microenvironment. Low pH was found to have a detrimental effect on both cell viability and matrix accumulation, which could be mitigated by priming cells using TGF- $\beta 3$. Investigating the activation of the transmembrane acid-sensing ion channels (ASIC-1 and -3) showed an increased expression of ASIC-1 in BMSCs and ASIC-3 in ACs at lower pH levels post-priming. Metabolic activity in terms of lactic acid production was also found to be affected significantly by priming, whereas oxygen and glucose consumptions did not change considerably. Overall, the study demonstrated that cells could be equipped to sustain the harsh environment of the IVD and promote accumulation of NP-like matrix through priming. Such an approach may open new avenues to engineer tissues capable of sustaining challenging microenvironments such as those found in the IVD.

Keywords: Acid-sensing ion channel, articular chondrocytes, bone-marrow-derived stem cells, hydrogel, metabolism, nucleus pulposus, pre-culture, transforming growth factor $\beta 3$.

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List of abbreviations

AC	articular chondrocyte	FBS	foetal bovine serum
AmpB	amphotericin B	GCR	glucose consumption rate
ASC	adipose stem cell	GDF	growth/differentiation factor
ASIC	acid-sensing ion channel	hg-DMEM	high-glucose Dulbecco's modified Eagle's medium
BMP	bone morphogenetic protein	IPSC	induced pluripotent stem cell
BMSC	bone-marrow-derived stem cell	IVD	intervertebral disc
DAPI	4',6-diamidino-2-phenylindole	LacA	lactic acid
DMMB	dimethylmethylene blue dye	lg-DMEM	low-glucose Dulbecco's modified Eagle's medium
	-binding assay	LPR	lactate production rate
ECM	extracellular matrix	NP	nucleus pulposus
EDTA	ethylenediaminetetraacetic acid	OCR	oxygen consumption rate

P/S	penicillin-streptomycin
PBS	phosphate-buffered saline
SCDM	supplemented chemically defined medium
sGAG	sulphated glycosaminoglycan
TGF- β	transforming growth factor β
TIMP	tissue inhibitors of metalloproteinase

Introduction

Low-back pain is a common global health burden, with an estimated annual cost of \$500 billion in the United States (Dieleman *et al.*, 2016; Manchikanti *et al.*, 2014). It is often associated with degenerative disc disease, which is characterised by degeneration of the NP, the central gelatinous tissue of the IVD (Vergroesen *et al.*, 2015). Given its avascular nature, the IVD poses a challenging microenvironment, with low levels of oxygen and glucose, which can exacerbate during IVD degeneration (Buckley *et al.*, 2018). A compounding issue also occurs during ageing, with the cartilage endplates becoming less permeable due to endplate calcification, which impedes the diffusion and nutrient exchange between the vertebral marrow and the disc itself (Grunhagen *et al.*, 2011). This results in a more hostile microenvironment due to limited nutrient transport, such as oxygen and glucose, as well as accumulation of metabolic by-products, such as LacA. This change in microenvironment results in increased acidity within the IVD, with values as low as 5.7–6.8 (severe to mild degenerated disc) (Diamant *et al.*, 1968; Nachemson, 1969), which affects cellular gene expression, proliferation and cell viability (Naqvi and Buckley, 2016; Razaq *et al.*, 2003; Wuertz *et al.*, 2009), leading to an imbalance in matrix anabolism and catabolism.

Acidic microenvironments affect cells through different intracellular pathways. Transmembrane ASICs are proton-gated sensitive channels and are expressed in various cell types, including ACs and NP cells (Gilbert *et al.*, 2016; Rong *et al.*, 2012; Zhou *et al.*, 2015). Both ASIC-1 and ASIC-3 subunits are expressed in NP cells and ACs, which are permeable to Na⁺ and Ca²⁺ ions (Cuesta *et al.*, 2014; Zhou *et al.*, 2015). When activated, the influx of ions triggers ion-dependent proteases, which can alter gene expression and induce cell apoptosis (Yuan *et al.*, 2016).

In the context of administration of cell-based therapies for disc regeneration, it is important to consider the microenvironment and ability of transplanted cells to function normally. Various cell types such as disc-derived cells (AF and NP cells), BMSCs and chondrocytes have been investigated for disc regeneration (Gorensek *et al.*, 2004; Illien-Junger *et al.*, 2012). Commercial entities including DiscGenics Inc. (Salt Lake City, UT, USA) and Mesoblast Ltd. (Melbourne, Australia) are also developing allogeneic-based products using NP cells and stem cells, respectively, and are advancing with clinical trials. ISTO Technologies (NuQu®, St.

Louis, MO, USA) previously explored the use of juvenile ACs combined with a fibrin carrier for disc repair; however, a phase II study was terminated for undisclosed reasons and the company has indicated a change in direction in their product portfolio (Smith *et al.*, 2018).

The harvesting and use of disc cells for cell-based therapeutics remains a challenge. Firstly, the isolation of disc cells from healthy tissue bears the risk of inducing disc degeneration and raises ethical concerns. Cells isolated from degenerated discs or herniated tissue during surgical procedures appear to be compromised, with a decreased collagen type II accumulation capacity and an increased rate of senescence (Park *et al.*, 2001; Roberts *et al.*, 2006). Moreover, disc cells have limited expansion capacities and limited reproducibility due to mixed populations (Liebscher *et al.*, 2011; Park *et al.*, 2001; Roberts *et al.*, 2006), which has motivated the use of alternative cell types such as stem cells or cartilage-derived cells. Both cell types are more easily accessible, have good *in vitro* expansion ability and deposit matrix similarly to the NP tissue (Malonzo *et al.*, 2015; Roughley, 2004). Moreover, the impact of the harsh microenvironment of the degenerated IVD is an important factor that may influence the success of these cell therapy approaches. Injected cells may experience limited nutrition or low pH conditions, resulting in compromised outcomes of the therapy. A further concern relates to the cell metabolism and whether the degenerated microenvironment can sustain increased metabolic demands upon delivery of cells to the targeted site.

Ideally, the injected cells should be capable of sustaining the acidic microenvironment of the disc as well as having a slow metabolic rate to avoid exacerbating nutrient deprivation effects. One approach to improve viability in low pH microenvironments may be through tissue engineering of a protective ECM niche around the cells, by priming with growth factors (Naqvi *et al.*, 2018; Zhang *et al.*, 2011). In carcinogenic tissue, the pericellular matrix increases drug resistance, indicating its protective features for cancerous cells (Noguera *et al.*, 2012). For IVD regeneration, proposed growth factors to induce differentiation of stem cells towards a disc-like phenotype include GDF-5, GDF-6, BMP-2 and TGF- β 1 (Bach *et al.*, 2017; Clarke *et al.*, 2014; Colombier *et al.*, 2016; Gantenbein-Ritter *et al.*, 2011; Stoyanov *et al.*, 2011). These cytokines have demonstrated enhanced matrix deposition capacities in both BMSCs and ACs (Coleman *et al.*, 2013; Colombier *et al.*, 2016; Gantenbein-Ritter *et al.*, 2011; Murphy *et al.*, 2015; Stoyanov *et al.*, 2011) and enhanced expression of NP-cell-specific markers, indicating successful differentiation towards an NP-cell phenotype (Clarke *et al.*, 2014). However, culture conditions vary in different studies and do not simulate the harsh IVD-like microenvironment (*i.e.* low oxygen and low glucose levels). Mueller *et al.* (2010) observed a slight benefit

using TGF- β 3 instead of TGF- β 1 within the first 14 d of chondrogenesis; however, the culture conditions were also not representative of the IVD. Previous studies have demonstrated that priming of BMSCs using TGF- β 3 promotes higher levels of sGAG and collagen deposition and supports cell survival of cryopreserved cells (Naqvi and Buckley, 2015a; Naqvi et al., 2018), which motivated the use of TGF- β 3 in the present study. Primed or pre-differentiated MSCs have been proposed in several other investigations for cartilage, bone and IVD regeneration (Grayson et al., 2010; Lam et al., 2014; Naqvi et al., 2019; Noth et al., 2007), making this strategy a promising approach to overcome microenvironmental challenges for disc repair.

The overall objective of the study was to explore the effects of acidic microenvironments on BMSCs and ACs and to determine if priming could enhance cellular response in low pH conditions in terms of viability, metabolism and disc-like matrix accumulation.

Material and Methods

Ethics statement

Porcine tissue (NP, bone marrow and articular cartilage) was obtained from a local abattoir and did not require ethical approval.

Experimental design

The study investigated the effect of matrix acidity on BMSCs and ACs non-primed and primed using TGF- β 3 in an alginate-bead culture (Fig. 1). Cells were exposed to i) various acid culture conditions (pH 7.1, 6.8 and 6.5) for 3 weeks (non-primed) or ii)

standard pH 7.4 with growth factor supplementation for 2 weeks prior to an additional 3 weeks acidic culture conditions (primed). Preliminary studies were also performed in which BMSCs (with and without TGF- β 3) and NP cells (without TGF- β 3) were pre-cultured for 2 weeks prior to exposure to pH 6.8 (low pH). All groups were maintained at 5.5 mmol/L glucose and 5 % oxygen for the duration of the experiment.

Cell isolation and monolayer expansion

Porcine NP cells were harvested from the lumbar region of porcine donor spines (3–4 months, 20–30 kg) within 3 h of sacrifice. NP tissue was enzymatically digested in 2.5 mg/mL pronase solution for 1 h followed by 2 h in 0.5 mg/mL collagenase solution at 37°C under constant rotation at 10 rpm (Stuart tube rotator). The digest was subjected to agitation cycles at the start and every 30 min thereafter, using a gentleMACS™ tissue dissociator (Miltenyi Biotech). Digested tissue/cell suspension was passed through a 100 μ m cell strainer to remove tissue debris followed by 70 μ m and 40 μ m cell strainers to separate notochordal cells from the desired NP cells. Then, cells were washed 3 times by repeated centrifugation at 650 $\times$ g for 5 min. Cells were seeded at 5×10^3 cells/cm² at 37°C and 5 % CO₂ in a humidified atmosphere in medium consisting of lg-DMEM (1 mg/mL D-glucose) supplemented with 10 % FBS, 100 U/mL penicillin, 100 μ g/mL streptomycin, 0.25 μ g/mL AmpB, 5 ng/mL FGF-2 (PeproTech, UK), and expanded to passage 2 (P2) with medium exchanges performed every 3 d.

Porcine BMSCs were isolated and maintained as previously described (Naqvi and Buckley, 2015a). Briefly, mononuclear cells were isolated from

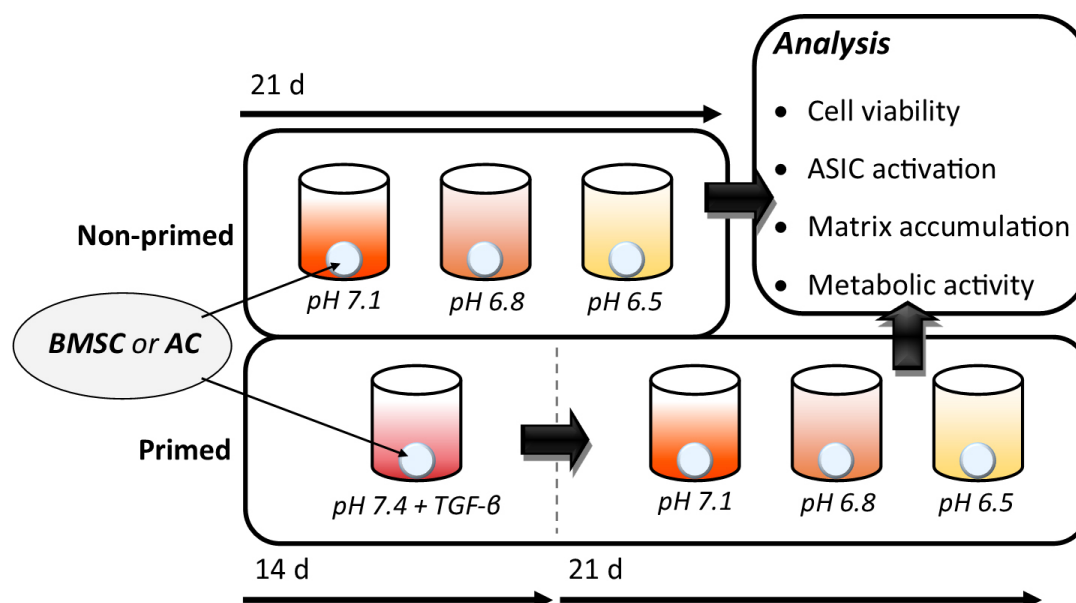


Fig. 1. Study design. Alginate-encapsulated BMSCs and ACs were cultured either in acidic culture conditions (pH 7.1, 6.8 and 6.5) without growth factor supplementation for 21 d (top, non-primed) or for 14 d with TGF- β 3 supplementation prior to 21 d of acidic culture (bottom, primed). Analysis was performed in terms of cell viability, ASIC activation, matrix accumulation and metabolic activity.

the femora of 3 month-old porcine donors and maintained in hg-DMEM (4.5 mg/mL D-glucose) supplemented with 10 % FBS, penicillin (100 U/mL), streptomycin (100 µg/mL) (all GIBCO) and AmpB (0.25 µg/mL, Sigma-Aldrich). Cells were washed with PBS after 72 h. When passaged, BMSCs were plated at 5×10^3 cells/cm² and expanded to P2 in a humidified atmosphere at 37 °C and 5 % CO₂.

Porcine ACs were isolated from donor-matching articular cartilage. Tissue was finely minced and digested at 37 °C for ~2.5 h under constant rotation at 10 rpm (Stuart tube rotator) using collagenase type II (3,000 U/mL, Gibco) in serum-free hg-DMEM containing P/S and AmpB (0.25 µg/mL). The digest was subjected to agitation cycles every 30 min, using a gentleMACS™ tissue dissociator (Miltenyi Biotech). Using a 100 µm cell strainer, debris was removed from the tissue/cell suspension. Cells were seeded at 5×10^3 cells/cm² at 37 °C and 5 % CO₂ in medium consisting of lg-DMEM (1 mg/mL D-glucose) supplemented with 10 % FBS, P/S and AmpB (0.25 µg/mL) and cultured until P1.

pH stabilisation

Media with 3 different pH values were prepared by adding an appropriate amount of LacA and HCl to supplemented lg-DMEM (1 mg/mL D-glucose, 308–340 mOs/kg). Briefly, LacA was added to the media to obtain physiological levels (4 mmol/L) normally found in the IVD (Bartels *et al.*, 1998). Additionally, different amounts of 3 mol/L HCl were added to adjust pH levels representative of different stages of degenerated disc conditions (*i.e.* pH 7.1, 6.8 and 6.5). The desired pH values were obtained after 24 h equilibration in a humidified incubator (CO₂-dependent) and were maintained for up to 72 h.

Alginate encapsulation and culture

Culture-expanded cells were encapsulated in 1.5 % alginate (w/v in PBS) (Pronova UP LVG, FMC NovaMatrix, Sandvika, Norway) at a density of 4×10^6 cells/mL. The alginate/cell suspension was passed through a 12 G needle and ionically crosslinked for 15 min using 100 mmol/L CaCl₂ (pH 7.2). For the primed group (pH 7.4), alginate beads were maintained in pH-adjusted SCDM, consisting of lg-DMEM, P/S, 1× insulin-transferrin-selenium (all from GIBCO), 0.25 µg/mL AmpB, 40 µg/mL L-proline, 1.5 mg/mL bovine serum albumin, 4.7 µg/mL linoleic acid, 50 µg/mL L-ascorbic acid-2-phosphate, 100 nmol/L dexamethasone (all Sigma-Aldrich) and 10 ng/mL TGF-β3 (PeproTech). Alginate beads were cultured in standard 24-well plates with one bead per well and 2 mL of supplemented medium in 5 % oxygen conditions with complete medium exchanges performed twice weekly. The pH value of acidic media was measured at each change, with no significant changes observed within that time period. Oxygen and glucose levels were chosen to replicate the native conditions within the IVD.

Live/dead assay

Cell viability was assessed using the LIVE/DEAD® Viability/Cytotoxicity assay kit (Invitrogen). Constructs were removed from culture, cut in half and incubated for 1 h at 37 °C in phenol-free lg-DMEM medium containing 2 µmol/L calcein-AM and 4 µmol/L ethidium homodimer-1 (Cambridge Bioscience, Cambridge, UK). Following incubation, constructs were imaged using a Leica SP8 scanning confocal microscope at 515 and 615 nm and analysed using Leica Application Suite X (LAS X) Software.

Biochemical analysis

Samples were digested with papain (125 µg/mL) in 0.1 mol/L sodium acetate, 5 mmol/L L-cysteine HCl, 0.05 mol/L EDTA and 55 mmol/L sodium citrate (Sigma-Aldrich) at 60 °C for 18 h followed by an additional incubation for 1 h with 1 mol/L sodium citrate under constant agitation to disrupt the alginate-calcium crosslinks. DNA content of each sample was quantified using the Hoechst Bisbenzimidazole 33258 dye assay, using a calf thymus DNA as standard. Proteoglycan content was estimated by quantifying the amount of sGAG in alginate beads using the DMMB assay (Blyscan, Biocolor Ltd., Carrickfergus, Northern Ireland, UK), with a chondroitin sulphate standard. Total collagen content was determined by measuring the hydroxyproline content. Samples were hydrolysed at 110 °C for 18 h in concentrated HCl (38 %) and assayed using a chloramine-T assay (Kafienah and Sims, 2004), using a hydroxyproline to collagen ratio of 1 : 7.69 (Ignat'eva *et al.*, 2007).

Immunohistochemistry and immunofluorescence

For evaluation of collagen types I and II, sections were treated for 1 h in a humidified environment at 37 °C with peroxidase followed by treatment with chondroitinase ABC (Sigma-Aldrich). Sections were incubated with goat serum to block non-specific sites and collagen type I (ab90395, 1 : 400; 1 mg/mL, mouse monoclonal, Abcam) or collagen type II (sc-52658, 1 : 400; 1 mg/mL, mouse monoclonal, Santa Cruz) primary antibodies were applied for 18 h at 4 °C. The secondary antibody (Goat Anti-Mouse IgG biotin conjugate, 1.5 : 200 for collagen type I and 1 : 300 for collagen type II; 2.1 mg/mL, B7151, Sigma-Aldrich) was added for 1 h followed by incubation with ABC reagent (Vectastain PK-400, Vector Labs, Peterborough, UK) for 45 min. Finally, sections were developed using DAB peroxidase (Vector Labs) for 5 min.

For evaluation of ASIC-1 (ACCN2) and ASIC-3, 5 sections per sample for ASIC-1 and ASIC-3 were heat-treated in a humidified environment at 37 °C using a citrate buffer (10 mmol/L in distilled water with 0.05% Tween 20, Sigma-Aldrich) at pH 6 or pronase (32 PUK/mL, Sigma-Aldrich), respectively. All sections were incubated with goat serum to block non-specific sites and anti ACCN2 (ASIC-1) (1 : 50; 1 mg/mL, ab176203, rabbit monoclonal,

Abcam) and anti ASIC-3 (1 : 50; 1 mg/mL, ab49333, rabbit monoclonal, Abcam) primary antibodies were applied for 18 h at 4 °C. The secondary antibody (ab15007, Goat Anti-Rabbit IgG H&L Alexa Fluor 488, 1 : 1,000; 2.1 mg/mL, Abcam) was added for 1 h followed by incubation with DAPI (Vectastain PK-400, Vector Labs, Peterborough, UK) for 10 min. 10 images per section were taken for image analysis.

Using ImageJ software, the raw integrated density (RawIntDen) of 10 cells per section was quantified and the background intensity by area subtracted to gain the corrected total cell fluorescence value.

Histology

At each time point, samples were fixed in 4 % paraformaldehyde overnight, dehydrated in a graded series of ethanol, embedded in paraffin wax, sectioned at 6 µm and affixed to microscope slides. The sections were stained with alcian blue/aldehyde fuchsin (30 min at room temperature) to assess sGAG content and picosirius red (1 h at room temperature) to assess collagen distribution (all Sigma-Aldrich).

Metabolic consumption rates

Oxygen consumption of non-primed and primed alginate beads was monitored for 3 d in air-tight glass vials using PreSens SensorVials and the SDR Sensor Dish® Reader system (PreSens Precision

Sensing GmbH, Regensburg, Germany). Lg-SCDM at specific acidity levels without growth factor supplementation was used. Glucose concentrations in medium samples from non-primed and primed groups were quantitatively measured using a glucose meter (FreeStyle Optimum, abbot Diabetes Care, Donegal, Ireland) (Naqvi and Buckley, 2015b). Lactate content was determined by measuring the reaction of lactate with NAD⁺ as well as lactate dehydrogenase with L-lactic acid as the standard, as described previously (Heywood *et al.*, 2006). All respiration rates (oxygen, glucose and lactate) were determined by normalising to the number of viable cells at each time point examined.

Statistical analysis

Statistical analysis was performed using GraphPad Prism (version 8). Three-way ANOVA was used for the analysis of variance with Tukey's multiple comparison tests to compare between groups (non-primed *vs.* primed, BMSCs *vs.* ACs and effect of pH). 3 technical replicates samples were analysed for each experimental group from 3 individual experiments with 3 different porcine donors (biological replicates). Numerical and graphical results are displayed as mean ± standard deviation and significance was accepted at a level of $p < 0.05$.

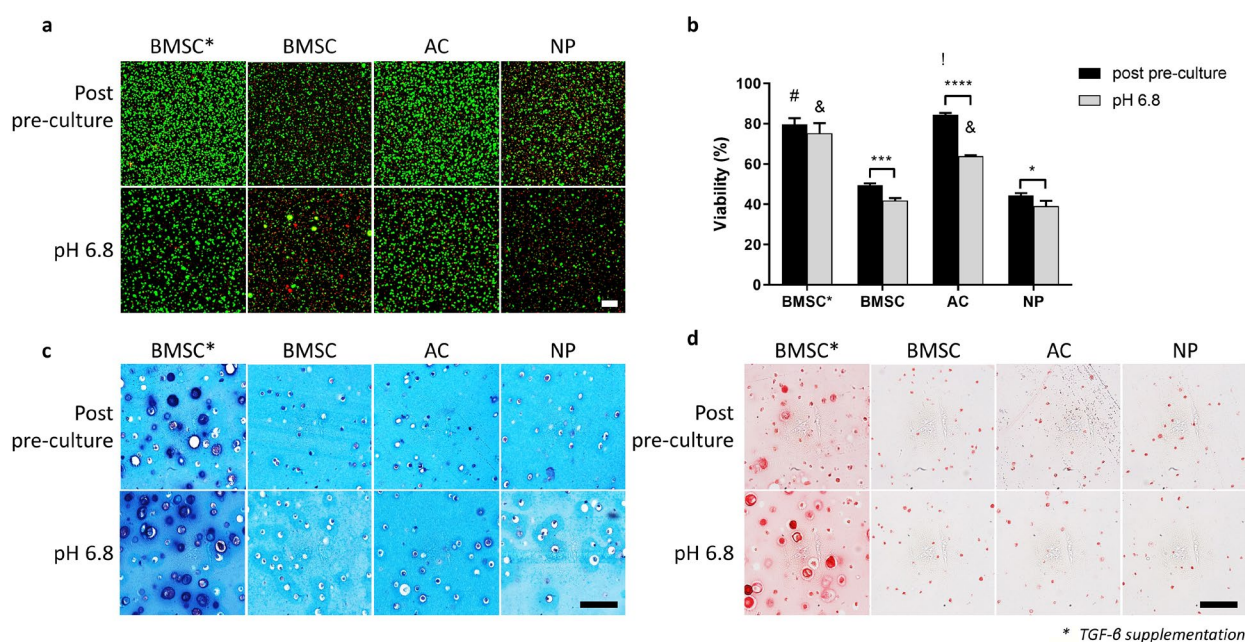


Fig. 2. Comparison of 14 d pre-culture of BMSCs, ACs and NP cells. (a) Live/dead images of BMSCs with TGF-β3 (indicated as BMSC*) as well as BMSCs, ACs and NP cells, all without TGF-β3, after 14 d pre-culture at pH 7.4 (top) and after an additional culture of 14 d at pH 6.8 (bottom). (b) Semi-quantitative analysis of cell viability after 14 d of pre-culture and after additional 14 d at pH 6.8. # indicates significant difference between post pre-cultured BMSC* when compared to BMSC and NP, respectively ($p < 0.0001$); & indicates significant difference between BMSCs and ACs after pH 6.8 culture compared to all other groups ($p < 0.0001$); ! indicates significant difference between ACs post pre-culture when compared to BMSCs and NP cells ($p < 0.0001$). *, *** and **** indicate significant differences between groups indicated ($p < 0.05$, $p < 0.001$ and $p < 0.0001$, respectively). (c) Alcian blue/aldehyde fuchsin staining indicating sGAG deposition (dark blue/purple) and (d) picosirius red staining indicating total collagen deposition. All scale bars: 150 µm.

Results

Pre-culture without TGF- β 3 resulted in decreased cell viability and limited matrix accumulation

Preliminary studies were performed in which BMSCs were pre-cultured for 2 weeks without TGF- β 3 prior to exposure to low pH at 6.8 for an additional 2 weeks (Fig. 2). Significantly lower cell viability was observed for BMSCs and NP cells cultured for 2 weeks at standard pH (7.4) without TGF- β 3, compared to BMSCs with TGF- β 3 (indicated BMSC*) and ACs ($p < 0.0001$) (Fig. 2a,b). Moreover, the viability of ACs dropped significantly after low-pH cell culture, despite the preculture at normal pH for 14 d. In addition, sGAG and collagen deposition was notably limited for cells cultured without TGF- β 3 (Fig. 2c,d), which motivated the study design to exclude pre-culture without growth factor and NP cells as experimental groups from further investigations.

Priming significantly improved BMSC viability

Baseline levels at day 0 showed homogeneously distributed viable cells across the construct for BMSCs and ACs, with a viability of 67.7 % and 75.5 %, respectively (Fig. 3a,b). Non-primed BMSCs exhibited low cell viability at all pH levels examined, with average values of 27.3 % and 20.7 % at pH 7.1 and 6.8, respectively, and a significant drop observed at pH 6.5 (2.2 %), compared to pH 7.1 ($p < 0.001$) and 6.8 ($p < 0.01$) (Fig. 3c,e). ACs, however, appeared to be less sensitive to different pH conditions, maintaining approximately 62.0 % viability across all pH levels investigated. After 14 d of priming using TGF- β 3, cell viability of both cell types was improved with a significant increase to 76.3 % and 73.5 % for BMSCs at pH 7.1 and 6.8, respectively ($p < 0.0001$). Notably, BMSCs at pH 6.5 showed a significant increase in cell viability of 62.0 %, which is a 28-fold increase when compared to non-primed conditions (Fig. 3d,e). ACs also demonstrated improved viability of 84.4 %, 80.7 % and 73.5 % at pH 7.1, 6.8 and 6.5, respectively. Quantification of total DNA showed a decreased level of DNA over time for non-primed beads compared to day 0, with consistently lower levels of DNA across all pH levels and cell types examined. 2 weeks of priming increased the amount of DNA significantly ($p < 0.0001$), which was maintained during culture in low pH medium for all groups examined (Fig. 3f).

Increased expression of ASIC-1 after priming of BMSCs

Semi-quantitative image analysis of ASIC expression demonstrated that pH and priming significantly correlated with ASIC-1 fluorescence signal ($p < 0.0001$), with no significant correlation observed for cell type and ASIC-1 expression ($p = 0.5042$) (Fig. 4a,b). Multiple analysis revealed significant differences between individual groups, including comparisons between non-primed cells at pH 6.8 ($p < 0.001$), primed cells at pH 7.1 and primed cells at pH 6.5 ($p < 0.0001$ and $p < 0.01$, respectively) (Fig.

4b). Expression level of ASIC-3 was also found to be significantly influenced by pH and priming ($p = 0.0015$ and $p = 0.0003$, respectively), with no difference based on cell type ($p = 0.1003$) (Fig. 4a,c). However, multiple analysis also showed significant differences between individual groups, including primed cells at pH 6.8 and 6.5 ($p < 0.01$ and $p < 0.05$, respectively). Interestingly, ACs formed cell clusters in all acidic levels (2-5 cells per cluster) after priming (Fig. 4a).

Matrix acidity inhibited sGAG accumulation, which could be mitigated by priming

Increased acidity was found to inhibit sGAG accumulation of non-primed ACs and was particularly pronounced at pH 6.5. Unsurprisingly, non-primed BMSCs did not accumulate sGAG at any pH (Fig. 5a). However, after exposure to TGF- β 3 for 14 d, both BMSCs and ACs accumulated a baseline level of sGAG of $83.8 \pm 49.2 \mu\text{g}$ and $78.8 \pm 15.4 \mu\text{g}$, respectively, which increased 1.6-fold and 1.4-fold for BMSCs at pH 7.1 and 6.8. ACs accumulated an additional 30 % sGAG when compared to post-priming baseline level throughout the low pH exposure (7.1 and 6.8) of 3 weeks (Fig. 5a). No change in sGAG deposition was observed after the 21 d culture at pH 6.5 for both BMSCs and ACs. Comparing the two different cell types, it was evident that non-primed ACs had a larger sGAG accumulation capacity than BMSCs; however, primed BMSCs produced similar levels across all groups (Fig. 5a,b). Statistical analysis using three-way ANOVA confirmed the significant impact of priming on sGAG deposition ($p < 0.0001$). Histologically, non-primed BMSCs only exhibited background staining for alginate (light blue) but no staining for sGAG (dark blue/purple) and this result was comparable to day 0 histology, which also correlated with the biochemical findings. Non-primed ACs exhibited pericellular deposition of sGAG at all pH levels, however at pH 6.5, the sGAG level deposited was below the detection limit of the assay (indicated with "nd") but was still visible histologically (Fig. 5c left). Histological images obtained post-priming with TGF- β 3 (prior to low pH culture) revealed a significant amount of sGAG deposition for both BMSCs and ACs, as expected. In addition, BMSCs cultured at pH 7.1 and pH 6.8 exhibited intense staining after priming, which correlated with the biochemical quantification (Fig. 5c right).

BMSCs accumulated more collagen, with a larger amount of collagen II deposition

A low level of collagen deposition was observed for both non-primed BMSCs and ACs (Fig. 6). However, similar to the results for sGAG accumulation, an increase in baseline total collagen levels was observed for BMSCs ($71.2 \pm 15.5 \mu\text{g}$) and ACs ($60.0 \pm 13.6 \mu\text{g}$) post-priming (Fig. 6a). For ACs, these levels were maintained at all pH levels, with no significant changes observed for either total collagen or collagen

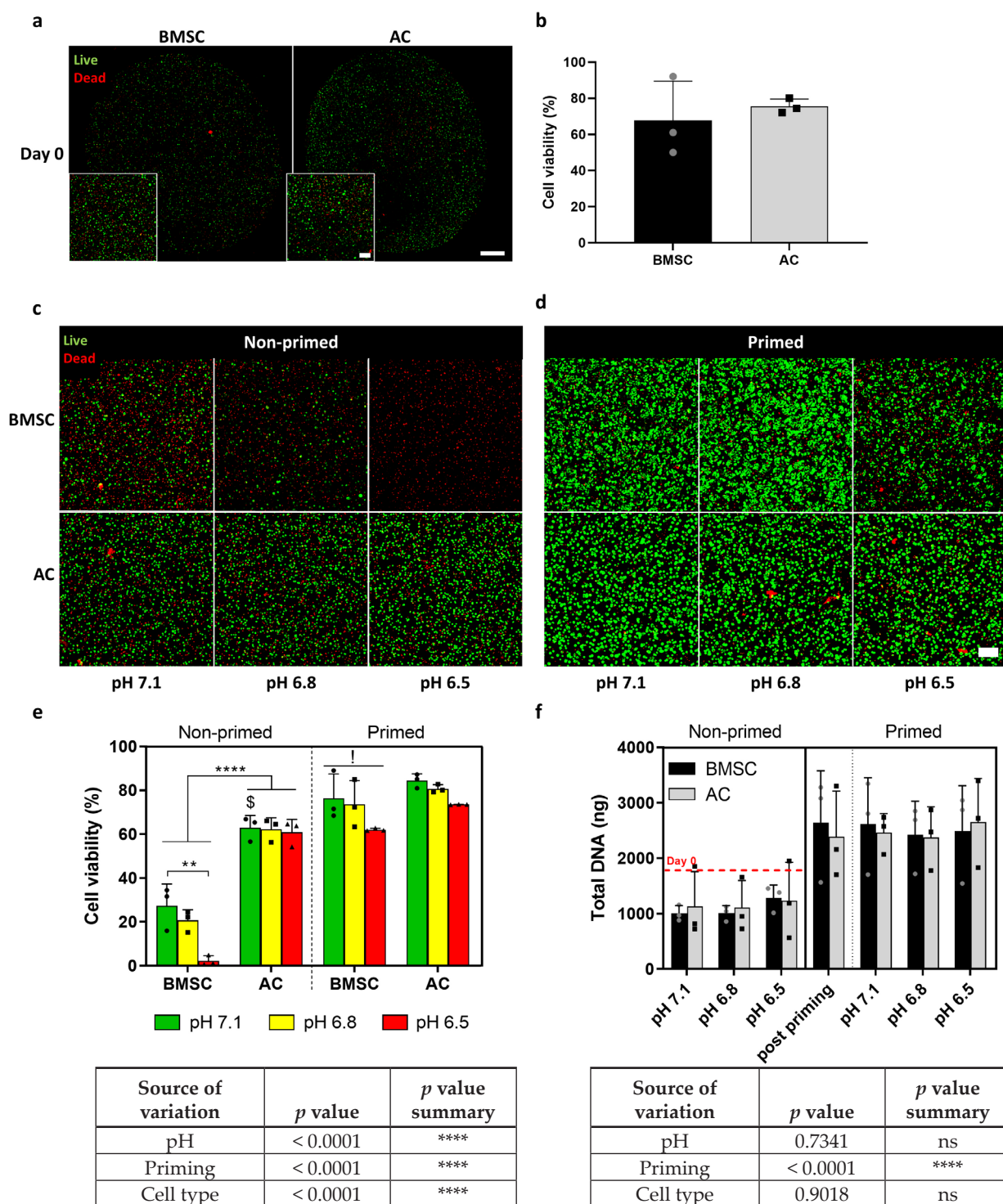


Fig. 3. Cell viability and proliferation of BMSCs and ACs. (a) Live/Dead images of BMSCs and ACs at day 0 (full construct, scale bar: 0.5 mm). (b) Semi-quantitative analysis of cell viability at day 0. (c) Live/Dead analysis after culture in different pH conditions without priming and (d) with priming. Scale bar: 150 μ m. (e) Semi-quantitative analysis of cell viability without priming and with priming. ! indicates significant difference between non-primed and primed BMSCs for all pH levels ($p < 0.0001$); \$ indicates significant difference between non-primed and primed ACs at pH 7.1 ($p < 0.05$); **** and ** indicate significant difference between specified groups ($p < 0.0001$ and $p < 0.01$, respectively). (f) Biochemical quantification of DNA content of non-primed and primed beads. Tables show results of three-way ANOVA of individual parameters on cell viability and total DNA, respectively. ns = not significant.

normalised by DNA content (Fig. 6a,b). However, BMSCs showed a 1.5-fold increase in total collagen when cultured for 21 d at pH 7.1 post-priming (Fig. 6a). This newly synthesised collagen by BMSCs was inhibited with increasing matrix acidity, with a pH of 6.5 resulting in maintenance of basal levels. Overall, three-way ANOVA revealed a significant impact of priming on total collagen and collagen/DNA level ($p < 0.0001$) as well as pH on collagen/DNA level ($p = 0.0043$). The choice of cell type was found to be significant for all parameters measured: total collagen ($p = 0.0104$), collagen/DNA ($p = 0.0002$) and sGAG/collagen ($p = 0.0032$). Biochemical results were confirmed histologically, exhibiting intense staining of primed samples prior to low pH culture

and after culture at pH 7.1 and 6.8, indicating a larger deposition of collagen matrix (Fig. 6d).

To evaluate matrix quality, the sGAG/collagen ratio was determined, with an average value of 1.5 ± 0.4 . A significant correlation was observed for sGAG/collagen ratio for priming and cell type ($p < 0.0001$ and $p = 0.0032$, respectively) but not pH level ($p = 0.6509$) (Fig. 6c). Weak staining for collagen type I was observed for both BMSCs and ACs for all pH levels after priming. Increased deposition of collagen type II was found in the pericellular region of all AC cultures for all acidic conditions. More intense and obvious collagen type II staining was detected throughout primed BMSC beads cultured at pH 7.1 and 6.8, which was diminished at pH 6.5 (Fig. 6d).

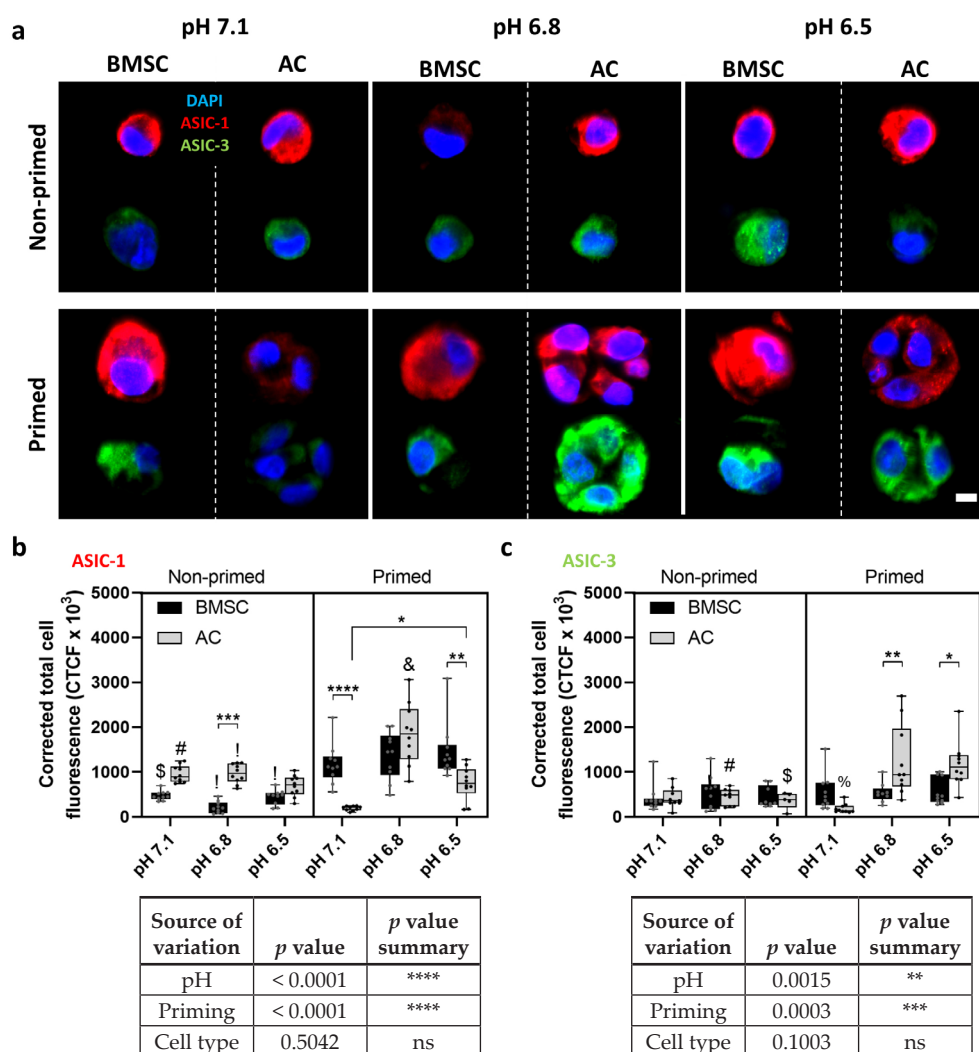


Fig. 4. Expression of ASICs in BMSCs and ACs. (a) Immunofluorescence of ASIC-1 (red) and ASIC-3 (green) of non-primed (top) and primed (bottom) BMSCs and ACs at different pH conditions (scale bar: 5 μ m). (b) Semi-quantitative evaluation of the intensity of ASIC-1 expression. \$, # and ! indicate significant difference between non-priming and priming of respective group ($p < 0.01$, $p < 0.001$ and $p < 0.0001$, respectively); & indicates significant difference between primed ACs at pH 6.8 when compared to primed ACs at all other pH levels ($p < 0.0001$); *, **, *** and **** indicate significant difference between specified groups ($p < 0.05$, $p < 0.01$, $p < 0.001$ and $p < 0.0001$, respectively). (c) Semi-quantitative evaluation of intensity of ASIC-3 expression. \$ and # indicate significant difference between non-priming and priming of respective group ($p < 0.01$ and $p < 0.001$, respectively); % indicates significant difference between primed ACs at pH 7.1 when compared to primed ACs at all other pH levels ($p < 0.0001$); * and ** indicate significant difference between specified groups ($p < 0.05$ and $p < 0.01$, respectively). Tables show results of three-way ANOVA of individual parameters on fluorescence intensity. ns = not significant.

Priming altered the lactate production of BMSCs and ACs

OCR of BMSCs was found to be upregulated (~2-fold) post-priming, whereas OCR of ACs tended to decrease post-priming (Fig. 7a). Overall, the cell type had a significant impact on OCR ($p = 0.0099$). There was a trend towards increased GCRs of BMSCs after priming at all pH levels; however,

the differences were not found to be statistically significant. ACs showed a trend towards decreased GCRs with increasing matrix acidity post-priming (Fig. 7b). A similar profile was observed for LPR, with a significant difference between non-primed and primed BMSCs at pH 6.8 ($p < 0.01$) as well as a trend towards decreasing LPR with increasing acidity in AC medium. Overall, three-way ANOVA revealed

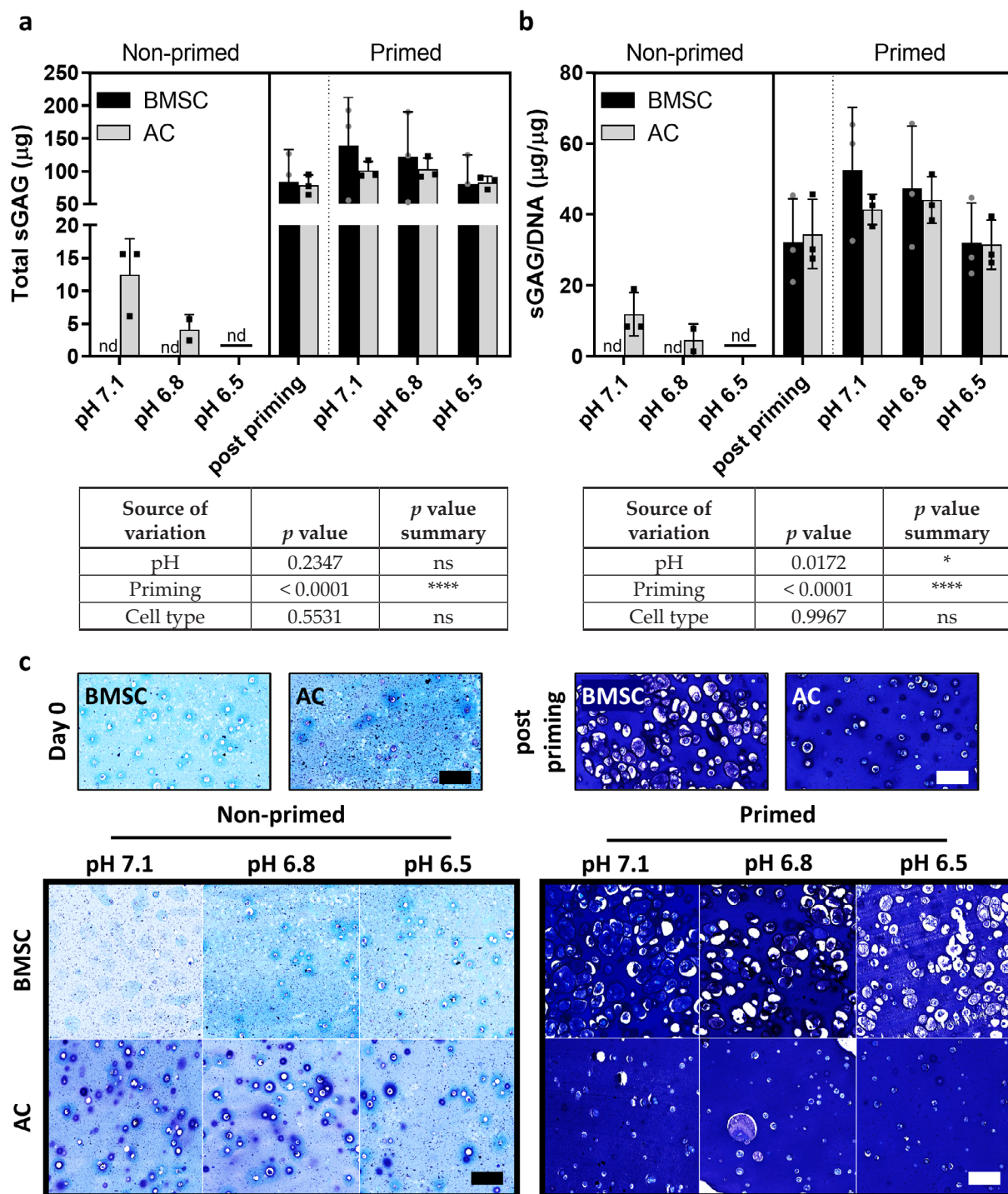


Fig. 5. sGAG accumulation of non-primed and primed BMSCs and ACs at different pH levels. (a) Total sGAG (μg) accumulation of non-primed or primed BMSCs (black bar) and ACs (grey bar) after 21 d of exposure to low pH conditions. (b) Normalised sGAG levels to total DNA ($\mu\text{g}/\mu\text{g}$); nd: not-detectable. Tables show results of three-way ANOVA of individual parameters on total sGAG levels and sGAG/DNA ratio, respectively. (c) Alcian blue/aldehyde fuchsin staining indicating sGAG deposition (dark blue/purple) of non-primed/primed cultures before and after low pH exposure. Scale bars: 100 μm . ns = not significant.

a significant correlation between LPR and priming ($p < 0.0001$) as well as pH ($p = 0.0091$) (Fig. 7c). No significant difference in LPR/GCR was found between individual groups; however, priming showed a significant impact overall ($p = 0.0147$) (Fig. 7d).

Discussion

It is widely believed that disc degeneration originates in the NP of the IVD, which is thought to become

more hostile with increasing degeneration due to nutrient transport limitations that can result in higher acidity microenvironments (Bibby *et al.*, 2005b; Huang *et al.*, 2014). If cell therapies are to become widespread as part of disc regeneration strategies, then it is important to ascertain whether transplanted cells can sustain these harsh microenvironments and to develop tissue engineering techniques to enhance cellular responses. The present study explored the effect of these challenging acidic conditions on BMSCs and ACs and investigated if priming could

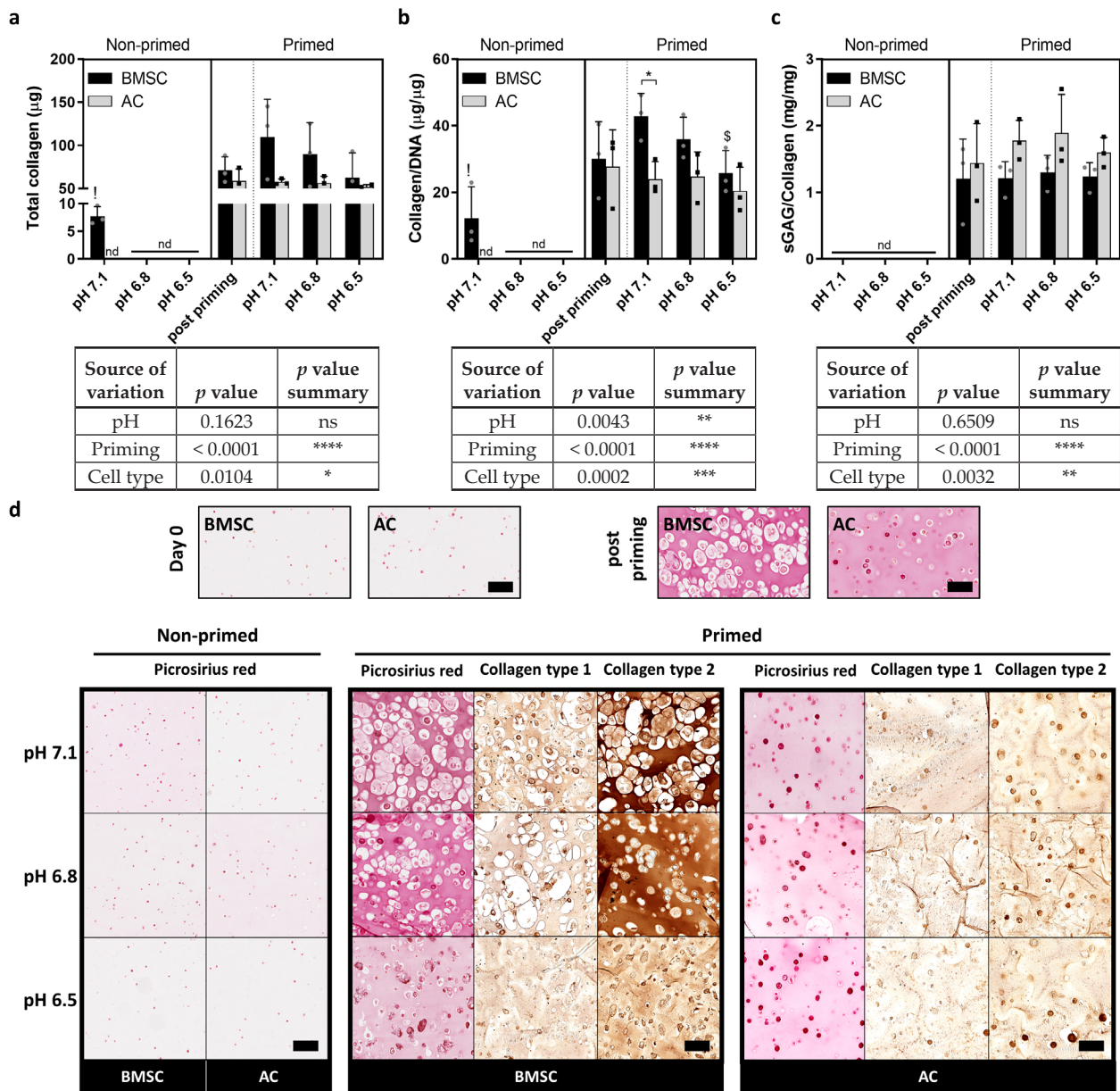


Fig. 6. Collagen accumulation of non-primed and primed BMSCs and ACs at different pH levels. (a) Total collagen deposition (μg) of non-primed and primed BMSCs (black bar) and ACs (grey bar) after 21 d. ! indicates significant difference between non-primed and primed samples of indicated group ($p < 0.0001$); nd: not-detectable. (b) Total collagen normalised to total DNA content ($\mu\text{g}/\mu\text{g}$). ! indicates significant difference between non-primed and primed samples of indicated group ($p < 0.0001$); * indicates significant difference between primed BMSCs and ACs at pH 7.1 ($p < 0.05$); \$ indicates significant difference between primed BMSCs at pH 6.5 to 7.1 ($p < 0.05$). (c) sGAG to collagen ratio (mg/mg). (d) Picrosirius red staining was used to visualise total collagen deposition at day 0, after priming phase and after low pH exposure. Further determination of the specific collagen types (I and II) was performed using immunohistochemical analysis. Scale bars: 100 μm . ns = not significant.

enhance cellular response in terms of viability, disc-like matrix accumulation and metabolism. By using TGF- β 3, the detrimental effects of low pH on cell viability and matrix accumulation could be overcome, demonstrating the potential of priming as a useful tool for cells that will be exposed to critical acidic conditions similar to those found in the degenerated IVD.

Previous studies have investigated the effects of an acidic pH on the viability of different cell types, including adipose-derived stem cells (Han *et al.*, 2014), BMSCs (Naqvi and Buckley, 2016; Wuertz *et al.*, 2009), NP mesenchymal stem cells (Han *et al.*, 2014), NP cells (Hodson *et al.*, 2018; Razaq *et al.*, 2003) and ACs (Razaq *et al.*, 2003), demonstrating decreasing viability and limited proliferation with increasing matrix acidity. This correlates with the findings in the non-primed groups of the present study.

However, none of the previous studies attempted to pre-culture cells to improve their resistance to acidic environments.

Given the significant interest in identifying alternative cell sources for disc repair, the potential benefits of priming BMSCs and ACs prior to acidic microenvironment exposure was examined. BMSCs and ACs are more easily accessible, exhibit better *in vitro* cell proliferation capacities when compared to disc cells and possess the ability to accumulate NP-like matrix (Le Maitre *et al.*, 2009; Malonzo *et al.*, 2015). Hence, BMSCs and ACs may represent alternative cell sources to disc-derived cells for cell-based repair of the IVD. After an initial preliminary study of pre-culture without TGF- β 3, lower cell viability and limited matrix deposition was observed when compared to culture with TGF- β 3. This motivated the exclusion of pre-culture without growth factor

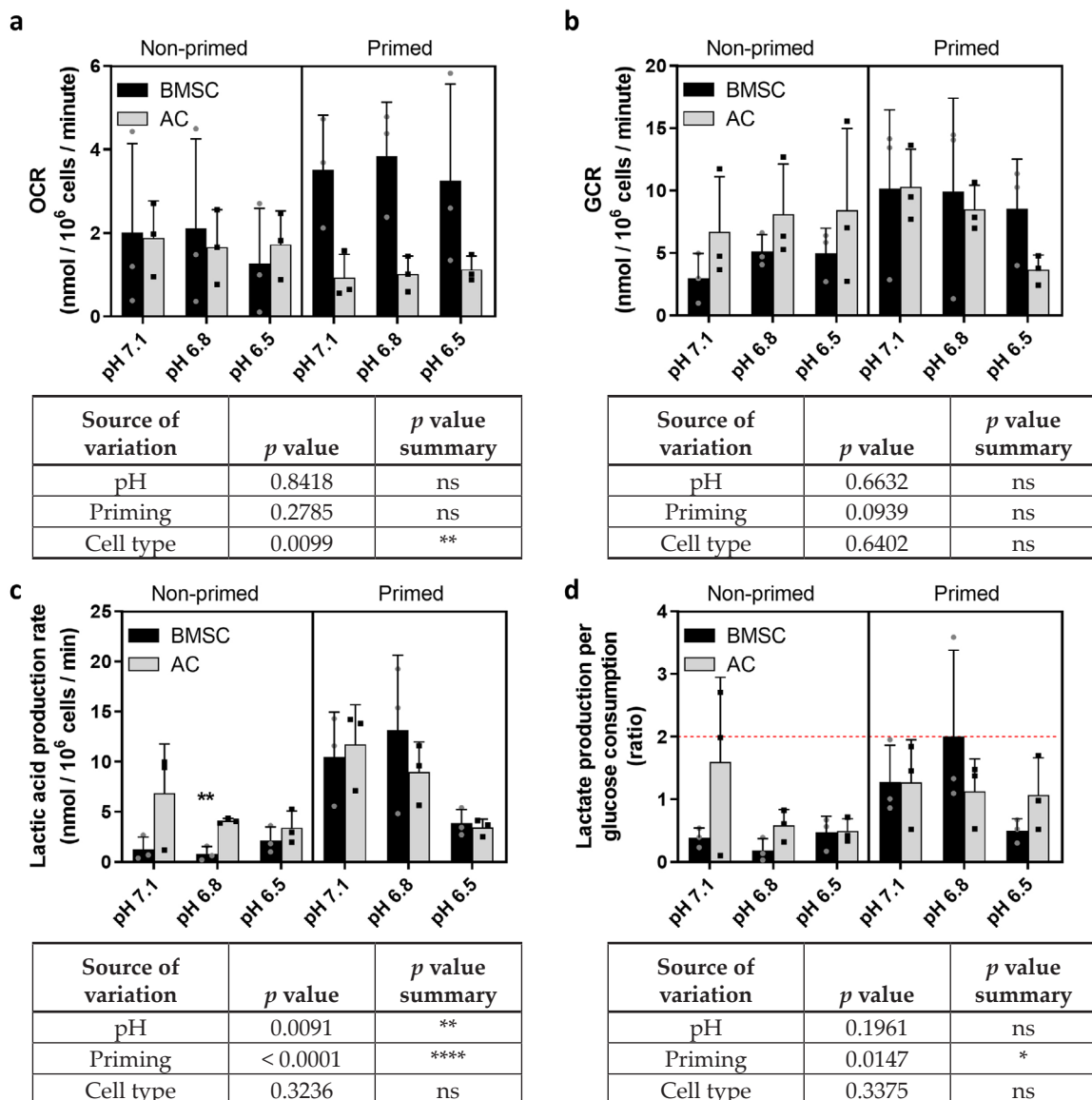


Fig. 7. Metabolic activity of non-primed and primed BMSCs and ACs. (a) OCR, (b) GCR and (c) LPR of BMSCs and ACs at different pH levels for non-primed (black bar) and primed (grey bar) in nmol/10⁶ cells/min. ** indicates significant difference between non-primed and primed samples of indicated group (*p* < 0.01). (d) Ratio of produced lactate per consumed glucose of cells either non-primed or primed at different media acidity. Tables show results of three-way ANOVA of individual parameters on metabolic output measures, respectively. ns = not significant.

supplementation and NP cells from the study. The primed groups using TGF- β 3 exhibited significantly higher viability after low pH culture, which may be due to the ECM being produced during the priming phase, providing a protective niche. Interestingly, a similar microenvironment (*i.e.* low pH, low oxygen) is found in tumours (Riemann *et al.*, 2013; Wei *et al.*, 2020), where cancer cells can dramatically proliferate despite these conditions. Comparing to tumour tissue, it has been reported that 60 % of its mass is ECM, including collagens and proteoglycans (Henke *et al.*, 2019), suggesting a correlation between ECM and cell survival in acidic conditions. The present study demonstrated that non-primed cells accumulated an inadequate amount of ECM molecules when exposed to acidic media. After priming in normal pH conditions, cells produced a large amount of sGAG, which was maintained after culturing in low pH media. Moreover, cell viability and proliferation for both primed cell types significantly increased, supporting the hypothesis that sGAG played a key role in the success of the priming strategy. A possible explanation could be a balance between the expression of the TIMPs, which are expressed during the priming phase (Leivonen *et al.*, 2013), and matrix metalloproteinases, which are released during the low pH culture (Gilbert *et al.*, 2016; Han *et al.*, 2014). This would result in maintenance of the pericellular matrix instead of degradation and explains the consistent levels of ECM surrounding primed ACs. In addition, sGAG can act as a proton conductor (Selberg *et al.*, 2019), which may guide free H⁺ ions within the acidic environment around the cells and, therefore, protect cells against direct contact.

In an attempt to better understand the mechanism behind the pH effect, the expression of ASICs was investigated. ASICs are expressed in various cell types, including neural cells, cancer cells, ACs and disc cells (Gilbert *et al.*, 2016; Yuan *et al.*, 2016; Zhou *et al.*, 2015), where they cause an influx of calcium and sodium ions into the cellular cytoplasm upon activation, which can result in downstream apoptotic effects (Rong *et al.*, 2012). However, in the present study, no such correlation was observed. In contrast, an increase in ASIC-1 expression after priming was found, concomitant with enhanced cell viability.

A previous study using tissue-culture plastic has demonstrated that the expression of this channel results in necrotic changes in rat cells (Zhou *et al.*, 2015), which disagrees with the present findings. However, the different culture technique, pH environment of 6.0, makes a direct comparison to the 3D alginate culture at pH between 7.1 and 6.5 (used in the present study) difficult. Additionally, in previous investigations of ASIC expression it has been found that ASIC-1, ASIC-2 and ASIC-3 can also be expressed at neutral pH (Gautschi *et al.*, 2017; Yu *et al.*, 2010), implying multiple routes by which those channels become transcribed and expressed. This suggested that in the present study a different mechanism enhanced ASIC expression.

Due to a different pathway activation mechanism, different intracellular processes may also be activated subsequently. Calcium, as a second messenger, is not only involved in inflammatory and apoptosis signalling but also in various pathways, regulating cell division, mechanotransduction and actin-reorganisation (Berridge *et al.*, 1998; Erickson *et al.*, 2003). Interestingly, a change in AC organisation after priming was observed, which exhibited a cluster formation of 2-5 cells within a lacuna, which is typical for hyaline cartilage. The NP has large amounts of proteoglycans and collagen, predominantly type II, similar to that of the hyaline cartilage tissue. To distinguish these two tissue types, Mwale *et al.* (2004) investigated differences in matrix composition of both and found that NP tissue contains a much higher sGAG to collagen ratio of 3.5 : 1 in comparison to a low ratio of 0.4 : 1 in hyaline cartilage. In the present study, a high level of collagen type II deposition in primed BMSCs was found, with a sGAG/collagen ratio of $1.5 \pm 0.4 : 1$ across all primed groups. This was not as high as native NP tissue but still 3.5-times higher than that of hyaline cartilage, illustrating the potential of priming to enhance NP-like matrix accumulation. Using TGF- β 3 for priming as a proof-of-principle has shown great improvements in terms of promoting cell viability; however, using a different growth factor such as GDF-5 or a combination may not only enhance cell viability but also further promote cells to deposit more NP-like matrix (Hodgkinson *et al.*, 2019; Le Maitre *et al.*, 2009).

Metabolic activity needs to be considered when proposing cell-based therapies for the IVD, given the limited nutrition available within the disc. In the present study, no significant correlation of priming on OCR or GCR was found by three-way ANOVA. However, the cell type was found to have a significant influence on OCR, highlighting its importance for disc repair. To compare with disc cells, NP cells from a degenerated disc have an OCR of 1.03-1.5 nmol/10⁶ cells/min (Cisewski *et al.*, 2018), which is similar to that of all non-primed samples. The tendency for increased OCR and GCR in primed BMSCs may be dependent on the donor cells and should be considered in terms of clinical translation, as higher rates will inevitably increase the nutrient demands of the implanted cells. Interestingly, priming was found to significantly influence LPR and pH level. Lactate production is highly influenced by surrounding pH, with decreasing levels at higher acidity (Bibby *et al.*, 2005a). This is in agreement with the present findings for primed cells as well as for non-primed ACs. Moreover, Pattappa *et al.* (2011) demonstrated increased lactate production by BMSCs during chondrogenic differentiation using TGF- β supplementation, which correlates with the results for primed BMSCs in the present study. However, non-primed cells produced lower levels of lactate for both cell types, demonstrating the different metabolic activity of primed and non-primed cells. During anaerobic glycolysis, cells produce approximately

2 moles of lactate for every mole of glucose consumed (Dashty, 2013), emphasising the challenges of cell nutrition within the IVD. Despite a significant impact of priming on the lactate to glucose ratio ($p < 0.05$), all groups showed a value below 2, except for primed BMSCs cultured at pH 6.8, suggesting that cells used aerobic glycolysis and experienced sufficient level of extracellular oxygen within the time period investigated. However, the elevated LPRs may raise concerns in delivering primed cells into the degenerated IVD, which could potentially cause further acidification of the microenvironment and adversely affect the resident disc cells. This could be balanced by reducing the number of primed cells introduced into the disc as part of the cell therapy. In addition, positive effects of co-culturing NP cells with BMSCs have been reported, showing improved matrix deposition capacities of NP cells (Chen *et al.*, 2017; Naqvi and Buckley, 2015a; Naqvi *et al.*, 2019; Richardson *et al.*, 2006), which may improve their resilience towards the decreasing pH and, therefore, support the strategy of using primed cells for disc repair.

There are several limitations associated with the present study. While cells from porcine donors are found to be similar to human cells, including *in vitro* expansion capacities, differentiation and immunomodulatory properties (Bosch *et al.*, 2006; Liu *et al.*, 2004; Ringe *et al.*, 2002; Thomson *et al.*, 1993), making them a suitable choice for initial proof-of-principle investigations, studies using human cells warrant further investigation to fully appreciate the effectiveness of priming strategies. In addition, while 3D hydrogel systems provide a better representation when compared to standard tissue-culture plastic, they cannot fully recapitulate the complex conditions that exist *in vivo*. Further studies will be necessary to investigate the effectiveness of priming cells using *in vivo* models. Moreover, future studies should examine the use of human cells to gain an appreciation of the effectiveness of priming cells for clinical translation. Ideally, for the non-primed group, the cells would be cultured for 14 d without TGF- β 3 supplementation, to directly compare ECM synthesis. However, based on the preliminary study, significant cell death and limited matrix accumulation of cells cultured without growth factors at normal pH were observed. BMSCs do not accumulate key matrix components without some form of growth factor stimulation (*i.e.* priming) and, therefore, the time period of culture is less of a concern. In addition, other growth factors such as GDF-5, GDF-6, BMP-2 and TGF- β 1 or combinations thereof should be explored to promote a stable disc-like phenotype. In summary, the important aspect of the present study was that it demonstrated the value of priming cells before they were subjected to harsh environments such as acidic pH, as doing so better protects cells in terms of viability. Therefore, priming could be an essential and important step towards successful clinical translation improving cell-based therapy outcomes.

Conclusion

The results of the present study showed that both BMSCs and ACs were negatively affected by low pH conditions in terms of cell viability and matrix accumulation, compromising their effectiveness for cell-based repair of tissues, exhibiting a challenging microenvironment. This effect could be overcome by priming using TGF- β 3 prior to low pH exposure. Priming was observed to enhance cell viability and provided a baseline level of ECM, offering a protective niche for subsequent matrix accumulation. Therefore, priming could be used as a powerful tissue engineering tool for cell-based regeneration of the IVD to help injected cells withstand its typical harsh microenvironment, facilitate deposition of *de novo* ECM components and help ameliorate degenerative effects.

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Discussion with Reviewers

Reviewer: Could the authors comment on how the specific cell type (BMSCs *versus* ASCs *versus* iPSCs) and the specific growth factor used may influence the success of priming? Have the authors considered integrating the mechanical microenvironment of the degenerate IVD into their model? How would this synergise with the biochemical microenvironment to affect stem cell priming?

Authors: The choice of cell type and growth factor supplementation is critical for different aims as this will determine the fate of the individual cell type. With respect to IVD regeneration, a cell type (BMSCs, ASCs, iPSCs or other differentiated cells such as ACs or nasal chondrocytes) that can sustain the harsh conditions typically experienced in the disc is desirable and a growth factor that promotes the deposition of a sGAG and collagen type II-rich ECM is highly favourable. The challenge with the non-homologous use of alternative cell types for disc repair is the regulatory framework and ensuring safety and efficacy. Growth factors from the TGF- β superfamily, which includes TGF- β , BMP and GDF, seem to be particularly suitable for promoting viability and matrix deposition. As the present study highlighted some advantages of using TGF- β 3 for priming, others have also shown great success using GDF5 and GDF6. A recent review by Hodgkinson *et al.* (2020, additional reference) summarises these successes, highlighting the potential for IVD repair and differentiation of MSCs towards an NP-like phenotype. Moreover, NP and AF cells express GDF6 receptors at different degeneration grades and show and increase in NP marker gene expression, such as aggrecan, keratin 18, keratin 19 and forkhead box F1, after GDF6 stimulation, indicating its potential (Hodgkinson *et al.*, 2020, additional reference). However, which growth factor is most efficient in promoting cell survival within the acidic environment is yet to be determined and their effectiveness may be dependent on the cell type or state of differentiation.

The integration of the mechanical microenvironment to this model would certainly provide a step towards recapitulating *in vivo* events and further the understanding of the interactions between mechanical and biochemical microenvironments and warrants further investigation.

Yongcan Huang: The acidic microenvironment was able to alter the biology of BMSCs and ACs. Would the activities of the transplanted cells change the situation of the hash degenerated disc microenvironment, making the disc ready for repair or regeneration?

Authors: This is a very interesting question and is important when considering cell-based therapies. It is unlikely that injected cells will improve the biochemical nutrient/acidic microenvironment *per se*. However, the introduction of BMSCs may have

a beneficial effect on the inflammatory environment and on the resident NP cell population, which could prove to be important. Also, the metabolic profile of transplanted cells is critical and a low nutrient demand in combination with high matrix deposition would be the most favourable scenario. Hence, if larger cell numbers are injected into the disc to boost the total amount of matrix being deposited, more cells will have to compete for nutrients and the acidity effect, as a result of glycolysis, would likely be exacerbated, compromising the effectiveness of the cell therapy. Computational modelling could potentially be used to help predict these effects and determine the optimal cell number for injection to repair disc tissue

without significantly impacting or adversely affecting the local nutrient microenvironment.

Additional Reference

Hodgkinson T, Gilbert HTJ, Pandya T, Diwan AD, Hoyland JA, Richardson SM (2020) Regenerative response of degenerate human nucleus pulposus cells to GDF6 stimulation. *Int J Mol Sci* **21**: 7143. DOI: 10.3390/ijms21197143.

Editor's note: The Scientific Editor responsible for this paper was Zhen Li.