

In Vitro Extracellular Matrix Accumulation of Nasal and Articular Chondrocytes for Intervertebral Disc Repair

S. Vedicherla^{1,2} and C.T. Buckley, Ph.D.^{1,2,3,4*}

¹ Trinity Centre for Bioengineering, Trinity Biomedical Sciences Institute, Trinity College Dublin, Ireland.

² School of Medicine, Trinity College Dublin

³ Department of Mechanical and Manufacturing Engineering, School of Engineering, Trinity College Dublin, Ireland.

⁴ Advanced Materials and Bioengineering Research (AMBER) Centre, Royal College of Surgeons in Ireland & 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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Key words: nasal cartilage; articular cartilage; oxygen; transforming growth factor beta (TGF- β)

Abstract

Chondrocyte based regenerative therapies for intervertebral disc repair such as Autologous Disc Cell Transplantation (ADCT, CODON) and allogeneic juvenile chondrocyte implantation (NuQu®, ISTO Technologies) have demonstrated good outcomes in clinical trials. However concerns remain with the supply demand reconciliation and issues surrounding immunoreactivity which exist for allogeneic-type technologies. The use of stem cells is challenging due to high growth factor requirements, regulatory barriers and differentiation towards a stable phenotype. Therefore, there is a need to identify alternative non-disc cell sources for the development and clinical translation of next generation therapies for IVD regeneration. In this study, we compared Nasal Chondrocytes (NC) as a non-disc alternative chondrocyte source with Articular Chondrocytes (AC) in terms of cell yield, morphology, proliferation kinetics and ability to produce key extracellular matrix components under 5% and 20% oxygen conditions, with and without exogenous TGF- β supplementation.

Results indicated that NC maintained proliferative capacity with high amounts of sGAG and lower collagen accumulation in the absence of TGF- β supplementation under 5% oxygen conditions. Importantly, osteogenesis and calcification was inhibited for NC when cultured in IVD-like microenvironmental conditions. The present study provides a rationale for the exploration of nasal chondrocytes as a promising, potent and clinically feasible autologous cell source for putative IVD repair strategies.

1 Introduction

The most common cause of lower back pain is intervertebral disc (IVD) degeneration with 97% of individuals over 50 years of age exhibiting degeneration of the IVD (Miller et al., 1988). Degenerative disc disease (DDD) is characterized by a series of deleterious changes in both cellular and structural integrity, leading to the loss of extracellular matrix (ECM) structure, altered biomechanical loading and symptomatic pain. Where ultimately the burden of back pain becomes debilitating to the point of surgical intervention, development of minimally invasive spinal surgical techniques, such as microdisectomy, have set a new standard. However where current techniques are successful at alleviating pain to some extent, they do not preserve or restore IVD function. While artificial disc and nucleus implants are effective in select patient populations, these surgical approaches are highly invasive and fail to address the underlying degenerative changes resulting in back pain. Wherein underlying cellular dysfunction results in functional loss, cellular-based implantation therapies have been proposed for the treatment of degenerated IVD with the aim of delivering cells to repopulate the nucleus pulposus (NP) region, augment tissue repair and restore functionality (Anderson et al., 2005; Benneker et al., 2014; Hohaus et al., 2008; Miot et al., 2012; Oehme et al., 2015).

Recent commercial approaches have extended beyond the use of culture-expanded Autologous Disc Chondrocyte Transplantation (ADCT, CODON, Germany) (Meisel et al., 2007) to allogeneic juvenile articular chondrocytes (ACs) injected with a commercially available fibrin gel (NuQu® Cell-Based Therapy, ISTO Tech., USA) (Coric D., 2013). Despite showing improvements in symptoms and functionality following lumbar discectomy for symptomatic disc herniation, concerns remain with the use of alternative non-disc chondrocytes or mesenchymal stem cells (MSCs) (Acosta et al., 2011). In the case of NuQu®,

issues still remain with supply and demand reconciliation using cadaveric juvenile chondrocytes and potential immunogenic reactions and rejection (Adkisson et al., 2010) which have not been elucidated for the disc microenvironment. Despite significant advancements in the development of commercial ventures such as ADCT and NuQu®, identification of an alternative cell source for robust regeneration is crucial to further advancement. While investigative trials continue for immunoselected adipose derived precursor cells (Mesoblast Ltd., Australia), additional growth factors (GFs) such as transforming growth factor-beta 3 (TGF- β 3) to induce and maintain chondrogenic differentiation (Acosta et al., 2011; Bobick et al., 2009) makes the use of adult MSCs challenging. Interestingly, within the degenerated human IVD, local progenitor cell populations have been identified previously (Risbud et al., 2007) and shown to exhibit slow cell proliferation kinetics in both annulus fibrosis and nucleus pulposus (Henriksson et al., 2009a) tissues. In direct comparison with bone marrow MSCs, NP progenitors were found to be phenotypically similar, although they demonstrated a lower propensity to undergo adipogenic differentiation (Blanco et al., 2010). The use of exogenous growth factors for in situ activation of these progenitors to orchestrate the repair of the IVD may perhaps be a feasible therapeutic strategy in the future.

However at the present time, given the regulatory challenges in the translational use of growth factors, the ability to optimize chondrogenesis through manipulation of environmental factors in the absence of exogenous growth factors is highly attractive. (Buckley et al., 2010; Freyria and Mallein-Gerin, 2012; Scotti et al., 2012; Twu et al., 2014). In addition, an important consideration is the harsh microenvironment within the degenerated intervertebral disc, which is characterised by reduced oxygen (Grunhagen et al., 2006) and reduced glucose concentration, (Bibby et al., 2005) creating a challenge in maintaining viable cellular

populations (Antoniou et al., 1996). For chondrocytes, tissue specific oxygen gradients have been shown to play an important role in maintaining tissue phenotype through the hypoxia inducible factor (HIF) family of nuclear regulatory elements (Malda et al., 2003) in a similar manner to TGF- β supplementation through MEK/ERK and PI3k/Akt pathways (Pratsinis et al., 2012; Risbud et al., 2005).

In exploring an alternative non-disc primary cell source for IVD regeneration, an ideal cell source should meet some of the following criteria: (i) autologous (ii) easily accessible (iii) sufficient cell yield (iv) capable of producing equivalent disc-like ECM in the absence of exogenous growth factor supplementation and (v) capable of sustaining the harsh microenvironment of the IVD. In addition, the selection of an appropriate biomaterial to provide a suitable 3D culture environment to support cell viability and matrix deposition is crucial to assess the regenerative capacity of cells. Among the natural polymers, alginate (Bron et al., 2011), agarose (Huang et al., 2004), atelocollagen (Sakai et al., 2003), fibrin (Colombini et al., 2014), collagen (Li et al., 2014), hyaluronic acid (Peroglio et al., 2012), gellan gum (Tsaryk et al., 2017) have been investigated for IVD regeneration *in vitro* and in animal models (Henriksson et al., 2009b; Li et al., 2014; Sakai et al., 2003).

Nasal septal cartilage, previously labelled as the pacemaker for orofacial cartilage tissue regeneration (Pelttari et al., 2014) could potentially provide for an attractive non-disc source of cells as it contains a large population of differentiated chondrocytes (Kafienah et al., 2002). Nasal chondrocytes (NCs) (Bujia, 1995; Kafienah et al., 2002; Richmon et al., 2005) have been used to engineer cartilaginous constructs both *in vitro* and *in vivo* (Asawa et al., 2009; Candrian et al., 2008; Chia et al., 2005; Fulco et al., 2014; Hellingman et al., 2011; Malda et al., 2004; Richmon et al., 2005; Rotter et al., 2001, 2002; Scotti et al., 2012; Tsaryk et al., 2017; Twu et

al., 2014; Watson and Reuther, 2014) and have recently been explored for nucleus pulposus tissue regeneration (Tsaryk et al., 2015; Tsaryk et al., 2017). However, the response of nasal chondrocytes to oxygen concentration and growth factors in maintaining phenotype and optimal NP-like matrix formation remains largely unknown in 3D culture. Therefore, the aim of this study was to evaluate the potential of nasal chondrocytes as an alternative cell source for IVD regeneration and to compare the relative response of articular and nasal chondrocytes in terms of cell viability and extracellular matrix accumulation when subjected to altered oxygen concentrations and exposure to the growth factor TGF- β 3.

2 Methods

2.1 Cell isolation and culture expansion

In accordance with institutional guidelines, donor matched skeletally mature, female bovine (mean age 18 months, n=3) hock joints and nasal septum were obtained from a local abattoir within 12 hours of sacrifice. For cell isolation, tissues were digested with collagenase type II (750U/ml at a ratio of 8ml per g of tissue) (Gibco, Ireland) for 12h. Cell yield and viability was determined with a hemocytometer and trypan blue exclusion. Cells were plated at 5×10^3 cells/cm² in T-175 flasks and maintained in Low Glucose–Dulbecco’s Modified Eagle Medium (LG-DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml)–streptomycin (100 mg/ml) and amphotericin B (0.25 mg/ml, (all Sigma-Aldrich). Cultures were expanded to passage one (P1) (7 days from initial isolation) in a humidified atmosphere at 37°C and 5% CO₂.

2.2 Cellular morphology and proliferation kinetics

Cells were fixed in 4% paraformaldehyde (PFA) and stained with hematoxylin and eosin (H&E) to assess cellular morphology. When subconfluent (~80%), cells were trypsinised and counted using trypan blue exclusion. The population doubling time was defined as the culture expansion time divided by the number of doublings during the expansion phase (Buckley and Kelly, 2012).

2.3 Alginate bead encapsulation and culture

Monolayer expanded NC and AC cells were trypsinised and 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 (\varnothing 5mm). Beads were maintained at 37°C with 5% CO_2 under normoxic (20% O_2) or physioxic (5% O_2) conditions. Chemically defined media (CDM) consisted of LG-DMEM supplemented with penicillin (100 U/mL)–streptomycin (100 μ g/mL), 0.25 μ g/mL amphotericin B, 40 μ g/mL L-proline, 1.5 mg/mL bovine serum albumin, 4.7 μ g/mL linoleic acid, 1 x insulin–transferrin–selenium, 50 μ g/mL L-ascorbic acid-2-phosphate and 100 nM dexamethasone (all Sigma-Aldrich) with or without TGF- β 3 (10ng/ml) (PeproTech, UK) supplementation. Each bead was maintained in 2mL of CDM in a 24 well culture plate with media changes performed twice weekly. Beads were assessed at days 0 and 21 in terms of cell viability, biochemical content and histological analysis.

2.4 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. Semi-quantitative analysis of LIVE and DEAD fractions were determined using IMAGEJ software (National Institutes of Health, Bethesda, Maryland, USA) and averaged over N=3 samples.

2.5 Quantitative DNA analysis

On removal from culture wet weight (ww) of the samples was recorded and constructs were frozen at -85 °C for further analysis. Samples were digested with 125 µg/mL papain in 0.1 M sodium acetate, 5 mM L-cysteine-HCl, 0.05 M ethylenediaminetetraacetic acid (EDTA), pH 6.0 (all from Sigma-Aldrich) at 60°C under constant rotation for 18 hrs followed by an additional incubation with 1M sodium citrate under constant rotation for 1 hour to disrupt the alginate calcium crosslinks. 20µl of the sample digest (300µl) was analysed in triplicate and DNA content was quantified using the Hoechst Bisbenzimidazole 33258 dye assay (Sigma-Aldrich) with a 20µg/ml calf thymus DNA standard (Kim et al., 1988) and normalised to tissue wet weight.

2.6 Quantitative sGAG analysis

40 µl of the papain digest was made up to 100 µl with papain buffer extract (100mM sodium phosphate buffer/5mM Na₂EDTA (ethylenediaminetetraacetic acid), pH 6.0) and analysed in triplicate using a chondroitin sulphate standard derived from bovine trachea (100 µg/ml) made up to 100 µl. Proteoglycan content was quantified using the dimethylmethylene blue dye-binding assay (Blyscan, Biocolor Ltd., Northern Ireland) and normalised to tissue wet weight.

2.7 Quantitative Collagen analysis

Total collagen content was determined by measuring the hydroxyproline content. 40 µl of papain digest was made up to 100 µl with papain buffer extract (100mM sodium phosphate buffer/5mM Na₂EDTA, pH 6.0) and was analysed in triplicate using a 1mg/ml trans-4-hydroxy-L-proline (56250, Fluka) standard solution. Samples were hydrolysed at 110°C for 18 hrs in 12M HCl, subsequently dried overnight in a heating block and assayed using a chloramine-T assay (Kafienah and Sims, 2004). The total amount of collagen was calculated using with a hydroxyproline:collagen ratio of 1:7.69 (Ignat'eva et al., 2007).

2.8 Histology and immunohistochemistry

At each time point samples were fixed in 4% PFA in sodium cacodylate–barium chloride buffer overnight, dehydrated through 30, 50, 70, 80, 90, 100%, 100% ethanol series with a final xylene change before embedding in paraffin wax. Sections of 8 µm were obtained with a microtome (Leica RM2125RT, Ashbourne, Ireland) and affixed to microscope slides. Prior to staining, sections were dewaxed using xylene and rehydrated in 100 to 70% ethanol baths followed by distilled water. Sections were stained with aldehyde fuschin/alcian blue to assess sGAG content and picro-sirius red to assess collagen distribution (all Sigma-Aldrich).

Collagen types I and II were evaluated through immunohistochemistry. Briefly, sections were treated with peroxidase, followed by treatment with chondroitinase ABC (Sigma-Aldrich) in a humidified environment at 37°C to enhance permeability of the ECM. Sections were incubated with goat serum to block nonspecific sites and collagen type I (ab6308, 1:400; 1 mg/ml), collagen type II (ab3092, 1:100; 1 mg/ml) primary antibodies (mouse monoclonal, Abcam, Cambridge, UK) were applied for 18 h at 4°C. Next, the secondary antibody (Anti-Mouse IgG biotin conjugate, 1:200; 2.1 mg/ml) (Sigma-Aldrich) was added for 1 h followed by incubation with ABC reagent (Vectastain PK-400, Vector Labs, Peterborough, UK) for 45 min. Color was developed using the Vectastain ABC reagent followed by exposure to peroxidase DAB substrate kit. Positive and negative controls of bovine ligament and cartilage were included for each batch (Supplementary Fig 1). Sections were imaged with an Olympus IX51 inverted fluorescent microscope fitted with an Olympus DP70 camera.

2.9 Osteogenic Assay

To assess the propensity of cells to undergo osteogenesis an osteogenic assay was performed (Pittenger et al., 1999; Vinardell et al., 2009). Preliminary studies demonstrated that osteogenic cultures matured rapidly such that mineral callus was found to detach from the 2D 6 well culture dish after 7 days. Therefore we employed a modified shortened assay with a time point of 7 days for all osteogenic cultures. Monolayer cultures were maintained at 37°C with 5% CO₂ under normoxic (20% O₂) or physioxic (5% O₂) conditions. After 7 days of osteogenic culture, well plates were fixed with cold ethanol and stained with 1% Alizarin Red solution (Sigma-Aldrich) and imaged. Orange–red staining indicating calcium deposits were considered positive.

2.10 Statistical analysis

Graphical presentation and statistical analysis was performed using Graphpad Prism (version 5). Two-way ANOVA comparisons were performed to assess significance between all combinations of groups. Groups were subsequently analysed using a general linear model for analysis of variance with factors of cell source, TGF- β supplementation and culturing regime (20% or 5% oxygen), to assess interactions between these factors. Bonferroni post-tests for multiple comparisons were used to compare between conditions. Graphical results are displayed as mean \pm standard deviation. Significance was accepted at a level of $p < 0.05$. The entire experiment was replicated independently with tissues from two additional donors which confirmed the findings presented in this manuscript.

3 Results

3.1 Cell isolation and proliferation kinetics

A 50% higher cell yield was obtained with NC compared to AC ($P < 0.0001$) (Fig. 1A). NC cells exhibited a shorter population doubling time compared with AC ($P < 0.0052$) (Fig. 1B) and this was affirmed through histology where more cells were observed in NC cultures compared to AC. Additionally, on histological evaluation, the morphology of NC cell populations was found to be heterogeneous comprised of different shaped cells (elongated, rounded, flat) compared with AC cultures in 2D (Fig. 1C).

3.2 DNA content and cell viability in 3D alginate hydrogel culture

NC cells trended towards higher DNA content compared with AC across all conditions (Fig. 2A). Low (5%) oxygen, with and without the presence of TGF- β enhanced proliferation for NC compared to AC cells ($P < 0.0001$). For AC cells, TGF- β was found to be beneficial under normoxic conditions ($P < 0.0001$) but under physioxia conditions, TGF- β supplementation was found to be detrimental with increased cell death ($P = 0.0018$). This was further supported by LIVE/DEAD staining (Fig. 2B). Semi quantitative analysis revealed the highest number of dead cells observed for NC, when cultured in low oxygen in the presence of TGF- β , and found to be equivalent to the number of viable cells. In contrast, under the same conditions, NC exhibited the highest number of viable cells when compared to AC ($P < 0.006$), (Fig. 2C).

3.3 Sulphated glycosaminoglycan (sGAG) accumulation in response to altered oxygen conditions and growth factor supplementation

Histological evaluation demonstrated that NC accumulated the highest amount of sGAG across all microenvironmental conditions (Fig. 3A). As confirmed by biochemical findings, NC beads accumulated the highest amounts of sGAG in both the presence ($P < 0.0001$) and absence of TGF- β ($P < 0.0001$) in response to physioxia compared to AC cultures (Fig. 3B). Under normoxic conditions, NC beads accumulated higher sGAG content compared to AC with ($P < 0.031$) and without ($P < 0.041$) TGF- β supplementation. In addition, TGF- β supplementation was found to enhance sGAG accumulation for both AC ($P < 0.0001$) and NC ($P < 0.0081$) beads compared to controls (without TGF- β supplementation). Similar results were observed when normalised on a per cell basis (Fig. 3C).

3.4 Collagen accumulation in response to altered oxygen conditions and growth factor supplementation

Overall, low collagen accumulation was observed for NC, (Fig. 4A) which was further reduced under physioxic conditions as confirmed through biochemical analysis (Fig. 4B). AC beads exhibited higher collagen content compared to NC when cultured in normoxic conditions in the presence of TGF- β ($P < 0.0001$). Overall, under normoxic conditions, TGF- β supplementation was found to enhance collagen accumulation for both AC ($P < 0.0014$) and NC ($P < 0.042$) beads compared to controls (without TGF- β supplementation), (Fig. 4B). Physioxia appeared to inhibit collagen accumulation for both AC and NC with no differences observed between cell type in the presence or absence of TGF- β supplementation (Fig 4B). Similar results were observed when normalised on a per cell basis, with the exception of AC cultures exhibiting higher collagen content compared to NC when cultured in physioxic conditions without TGF- β supplementation ($P < 0.0012$) (Fig. 4C). Taken together, the relatively low collagen accumulation for NC with high sGAG content resulted in higher sGAG-collagen ratios when cultured under physioxic conditions, even in the absence of TGF- β supplementation ($P < 0.0001$) (Fig. 4D). Furthermore, NC beads cultured under physioxic conditions without TGF- β supplementation exhibited a higher sGAG-collagen ratio compared to NC beads culture in the presence of TGF- β ($P < 0.0401$).

3.5 Relative Collagen types I and II in response to altered oxygen conditions and growth factor supplementation

In general, collagen type I accumulation was found to be relatively low across all groups. For AC, TGF- β supplementation appeared to enhance collagen type I under normoxic

conditions (Fig. 5A). Collagen type II was found to be enhanced with TGF- β supplementation and physioxia across both tissue types (Fig 5B). In addition, NC beads exhibited more intense staining for collagen type II compared with AC across all conditions.

3.6 Osteogenic propensity in 2D monolayer in response to altered oxygen conditions

As nasal chondrocytes have previously been shown to have a capacity to undergo endochondral ossification (Pippenger et al., 2015; Shafiee et al., 2011), we sought to assay the osteogenic propensity of AC and NC cells under different oxygen concentrations in 2D monolayer. Significant alizarin red staining was observed for NC cells cultured in normoxia in the presence of osteogenic factors, but not in physioxia conditions (Fig 5C). No staining was observed in the absence of osteogenic factors for either cell types (data not shown).

4 Discussion

In this study, we sought to determine the extracellular matrix accumulation by nasal and articular chondrocytes in response to altered oxygen concentration and growth factor stimulation when cultured in 3D alginate hydrogel beads. Overall, when cultured in physioxia (5% oxygen) conditions, simulating those found in native IVD tissue, NC exhibited improved proliferative capacity with higher amounts of sGAG and lower collagen accumulation compared to AC in the absence of TGF- β supplementation.

In line with several studies using human nasal chondrocytes, we observed that bovine nasal chondrocytes retain good matrix accumulation properties post expansion (Chia et al., 2005; Hellingman et al., 2011; Malda et al., 2004; Richmon et al., 2005; Rotter et al., 2002; Tay et al., 2004). Importantly, physioxia (5% oxygen) is a potent environmental modulator of

proliferation and matrix accumulation as demonstrated previously (Buckley et al., 2010; Twu et al., 2014). Even in the absence of TGF- β , physioxia was found to enhance extracellular matrix of NC compared with AC. This could be attributed to the lack of PPAR signalling in NC which hampers signalling in AC and necessitates exogenous TGF- β stimulation (do Amaral et al., 2012). A major factor limiting the use of autologous chondrocyte based therapies is dedifferentiation which is associated with extensive laboratory-based expansion and where growth factor supplementation is found to be necessary to maintain or restore phenotype (Gruber et al., 1997). The ability to modulate oxygen concentration to maintain phenotypic expression in nasal chondrocytes in the absence of TGF- β may make them an attractive cell source for IVD repair. Buckley et al. previously demonstrated that TGF- β supplementation in low oxygen (5% O₂) conditions was detrimental for agarose encapsulated porcine articular chondrocytes resulting in increased cell death, reduced proliferation and matrix accumulation (Buckley et al., 2010). We observed similar results in the present work for bovine AC which could be attributed to the confounding interactions of 5% oxygen on the same pathways as suggested previously (Buckley et al., 2010).

Cell yield from NC was found to be higher compared with AC in line with previous studies (Hellingman et al., 2011; Pleumeekers et al., 2014; Tay et al., 2004). Although higher cell yields have been previously observed in auricular cartilage compared with NC, the ability to produce tissue in an age independent manner makes them an ideal cell source (Rotter et al., 2001, 2002; Tay et al., 2004). Minimal donor site morbidity with a nasal biopsy may facilitate the use of autologous cells and could circumvent concerns surrounding disease transmission, immune rejection and ethical considerations associated with allogeneic tissue (Marmotti et al.,

2013). Improved proliferative kinetics of NC cells, 25% greater than AC, is also advantageous with reduced time and costs involved in cell amplification.

Primary NC cultures were found to be morphologically different when cultured in monolayer. These morphological differences have previously been reported to be dependent on the layer in which they were isolated from, with an elongated population of surface chondrocytes and oval cell population from deep zone cartilage in 2D culture. Nasal cartilage is surrounded by a layer of perichondrium, which remains attached to septum cartilage during sample harvesting and this could explain the mixed population observed initially in 2D culture (do Amaral et al., 2012). However, when cultured in a 3D alginate system, these cells, even in the absence of exogenous growth factor supplementation were found to adopt a rounded morphology most likely due to the absence of integrin binding sites of alginate.

The presence of a progenitor population within the surface of juvenile articular cartilage with appositional growth to connective tissue and differentiation plasticity has previously been shown in both bovine and rabbit models (Dowthwaite et al., 2004; Shapiro et al., 1977). Similarly, the perichondrium cambium has been shown to contain a source of chondroprogenitors in ear and rib cartilage (Engkvist et al., 1979; Upton et al., 1981). Of note, surface derived nasoseptal chondrocytes express positive surface markers (CD44, CD73 and CD105) described for mesenchymal stem cells, with the exception of CD146, a perivascular cell marker (do Amaral et al., 2012). In this study, AC and NC were taken from digested whole articular and nasal cartilage tissue with collagenase type II, without stratification of subpopulation or purification. Hence, the role of progenitor subsets was beyond the scope of this study.

AC and NC chondrocytes appear to have differential propensity for depositing different collagen types, which is possibly due to their different anatomical origin. Where normoxia was seen to upregulate collagen synthesis in both AC and NC, AC exhibited a higher total collagen accumulation in response to TGF- β stimulation. On closer examination of immunohistochemistry staining differential amounts of collagen type I and type II were observed. TGF- β stimulation and 20% O₂ appears to be promoting higher levels of collagen type I deposition compared to NC and could account for the higher total collagen accumulation observed for AC groups. In addition, native healthy nucleus pulposus tissue has a high sGAG-Collagen ratio, (Mwale et al., 2004) giving rise to its unique biomechanical properties. This ratio is perhaps one for the best metrics in determining whether a candidate cell source is capable of producing NP-like tissue. The relatively high sGAG to collagen ratio of NC is comparable to the high ratio reported for NP in the literature (Mwale et al., 2004). Additionally, such matrix ratios were achieved for NC when cultured under physioxic conditions without TGF- β supplementation. This is an important observation to be considered and makes NC a relevant and suitable cell source for IVD regeneration strategies.

Nasal chondrocytes have been explored previously for their osteogenic capacity and applications in bone tissue engineering (Pippenger et al., 2015; Shafiee et al., 2011) which could ordinarily be a significant limitation for the clinical translation of nasal chondrocytes for disc repair. Indeed, we did observe a greater propensity of NC cells to undergo osteogenesis compared with AC when supplemented with osteogenic factors and cultured in normoxic conditions. However, physioxic conditions inhibited this tendency towards mineralization. Importantly, the disc microenvironment is characterised by low oxygen (5%-1%) (Bartels et al., 1998), low glucose (<5mM) (Bibby et al., 2005) and low pH conditions (Bartels et al., 1998; Grunhagen et al., 2006; Urban, 2002). Inhibition of mineralisation under low oxygen

conditions may be due to downregulation of ANK, a pyrophosphate transporter known to be involved in controlling mineralization, which is negatively regulated by both HIF1 and HIF2 (Skubutyte et al., 2010). Therefore, it is highly unlikely that the native disc environment could provide the necessary biochemical cues to promote osteogenesis, although this would need to be confirmed using animal models. Whether the findings of this work directly translate when using human nasal tissue warrants further investigation and validation. In addition, further studies addressing cellular response in the context of the biophysical stimulation and in vivo assessment of these approaches would be required to facilitate successful clinical translation.

In conclusion, depending on anatomical site of harvested tissue, we demonstrate the influence of microenvironmental factors such as oxygen concentration in regulating proliferation and functionality of chondrocytes even in the absence of growth factor signalling. Given its anatomical location and ease of accessibility for harvesting biopsies of tissue, nasal cartilage may provide an attractive autologous source of cells. This has expanded the role of nasal chondrocytes from facial cartilaginous structures (Fulco et al., 2014) to distant anatomical regions as knee articular cartilage defects (Mumme et al., 2016), paving the way for the use of NCs in IVD regeneration. In the present work we add to this emerging concept by demonstrating in vitro functionality using nasal derived chondrocytes compared to articular chondrocytes. The present study therefore provides a rationale and strengthens the case for the exploration of nasal chondrocytes as a promising cell source for putative IVD repair strategies.

Figure Legends

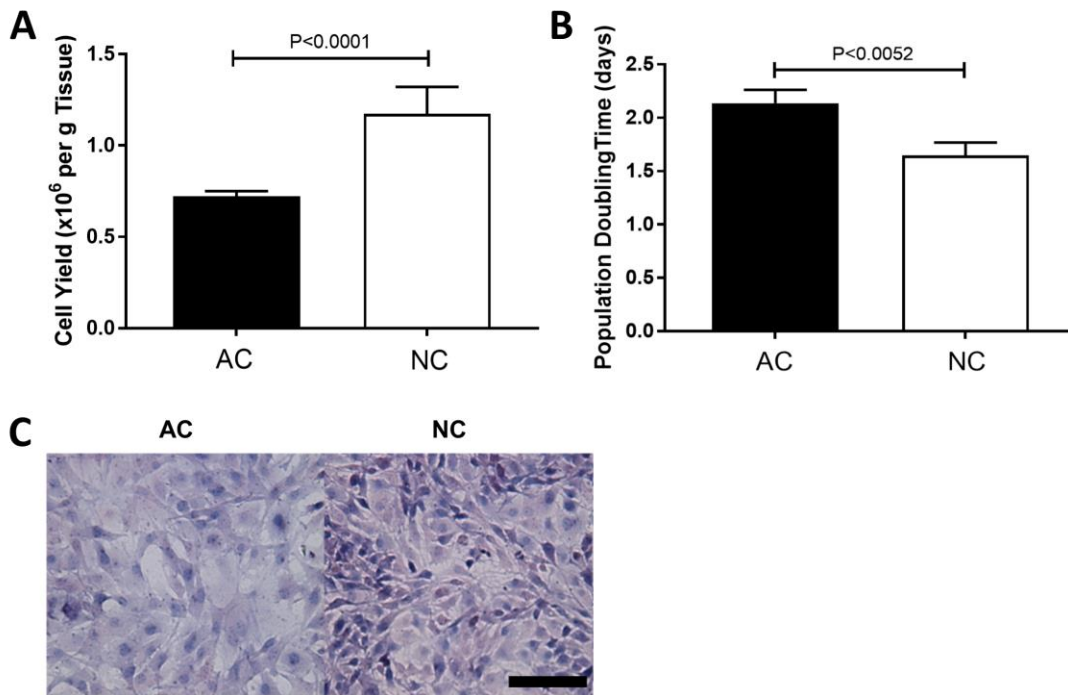


Figure 1

FIG. 1. Characterisation of Articular Chondrocytes (AC) and Nasal Chondrocytes (NC) isolated from cartilaginous tissues. (A) Cell yield normalised to wet weight (per gram) of tissue. (B) Population doubling time (days) for AC and NC cultures initially seeded at 5×10^3 cells/cm² in T-175 flasks. (C) AC and NC cells in monolayer stained with H&E following 7 days of expansion on six well culture plates seeded at an initial density 5×10^3 cells/well. Scale bar: 500 μm .

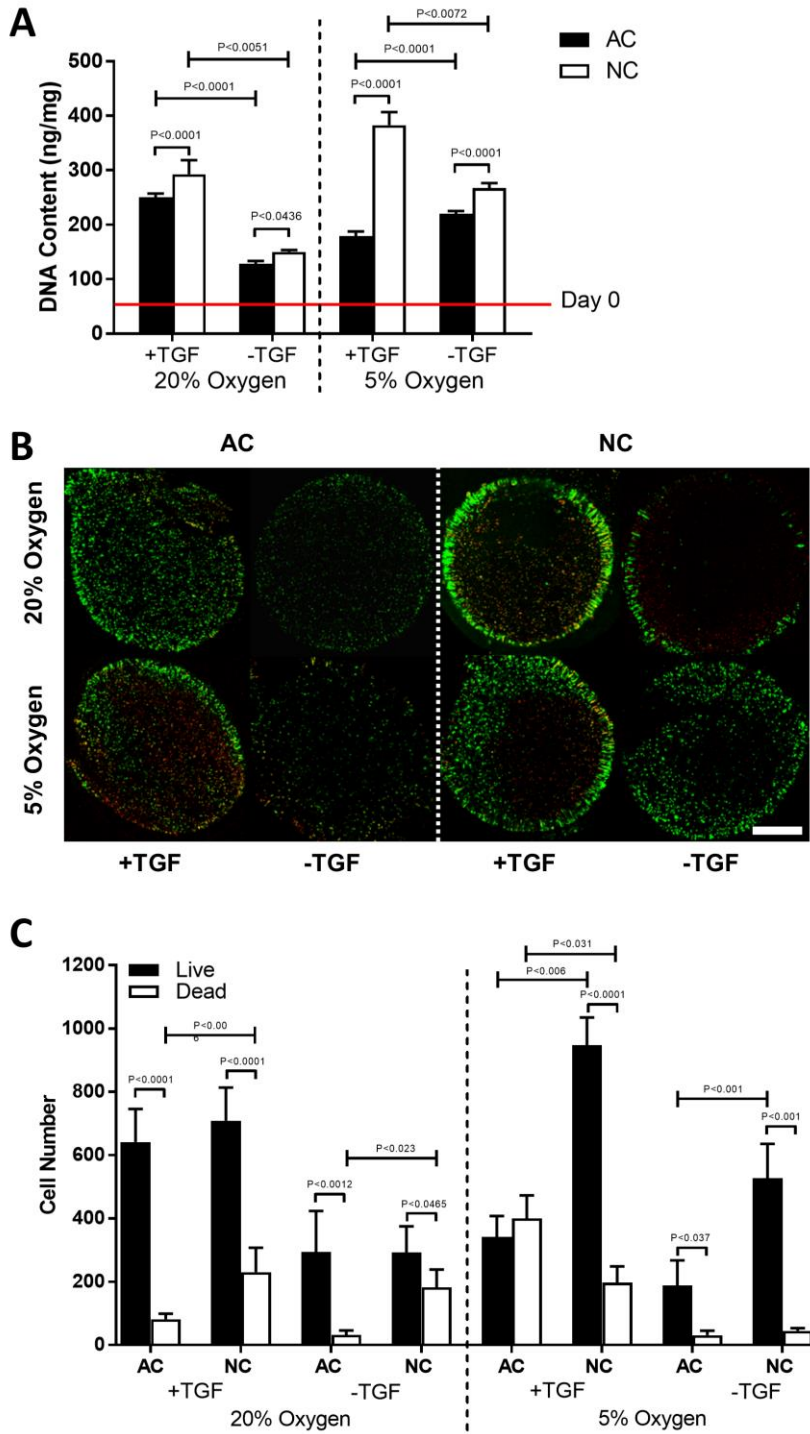


Figure 2

FIG. 2. DNA content and cell viability for Articular Chondrocytes (AC) and Nasal Chondrocytes (NC) after 21 days in alginate bead culture subjected to either 20% (normoxic)

or 5% (physioxic) oxygen concentration with or without TGF- β supplementation. (A) DNA content normalised to wet weight (ng/mg), red line indicates baseline values at day 0. (B) Cell viability for AC and NC alginate beads at day 21. Scale bar: 1 mm. (C) Semi-quantitative analysis and cell number for AC and NC alginate beads at day 21.

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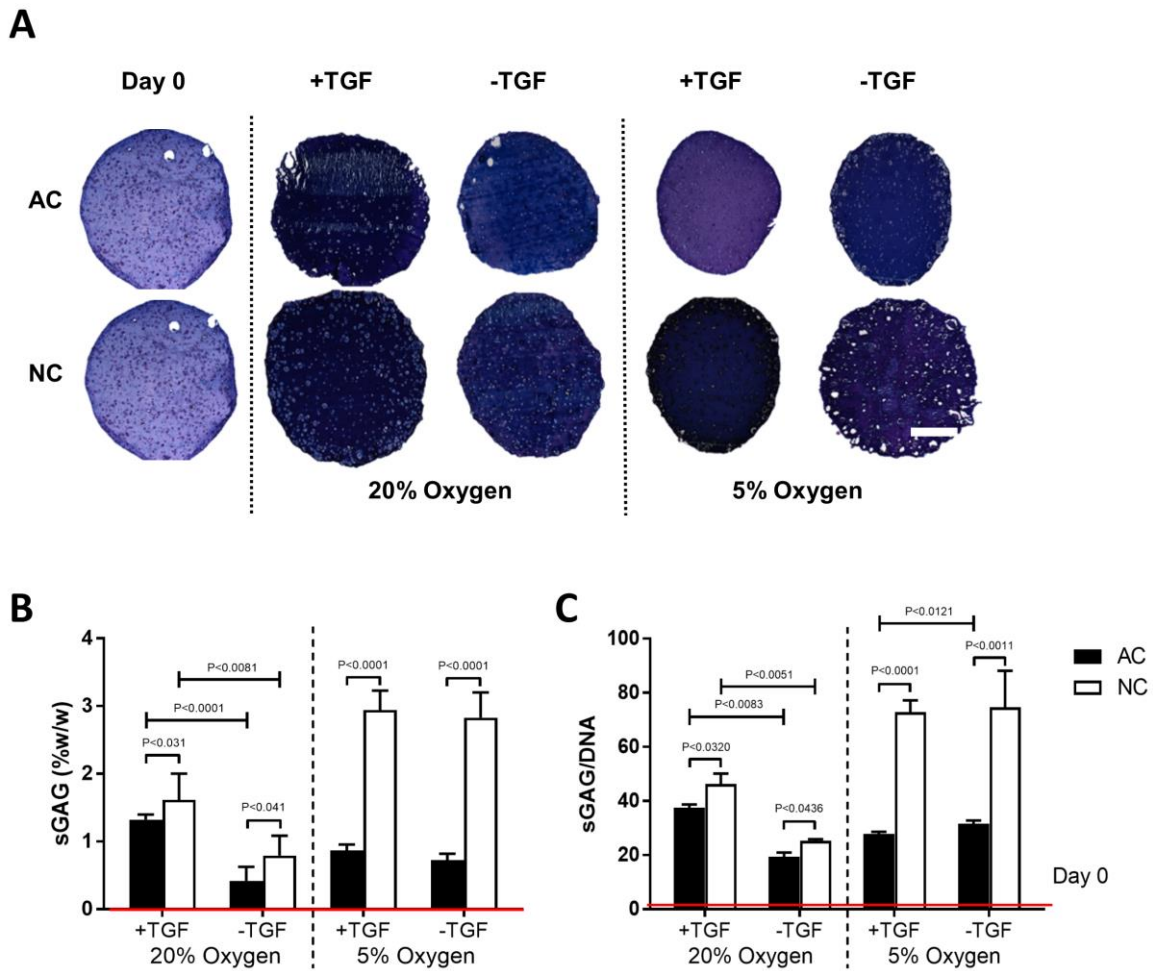


Figure 3

FIG. 3. sGAG accumulation for Articular Chondrocytes (AC) and Nasal Chondrocytes (NC) after 21 days in alginate bead culture subjected to either 20% (normoxic) or 5% (physioxic) oxygen concentration with or without TGF- β supplementation. (A) Histological evaluation with aldehyde fuchsin and alcian blue to identify sGAG at day 0 and day 21; deep blue/purple staining indicates sGAG accumulation and light blue staining indicates residual alginate. Scale bar: 1 mm (B) sGAG content normalised to percentage wet weight (%w/w) and (C) sGAG normalized on a per cell basis (sGAG/DNA). Red line indicates baseline values at Day 0.

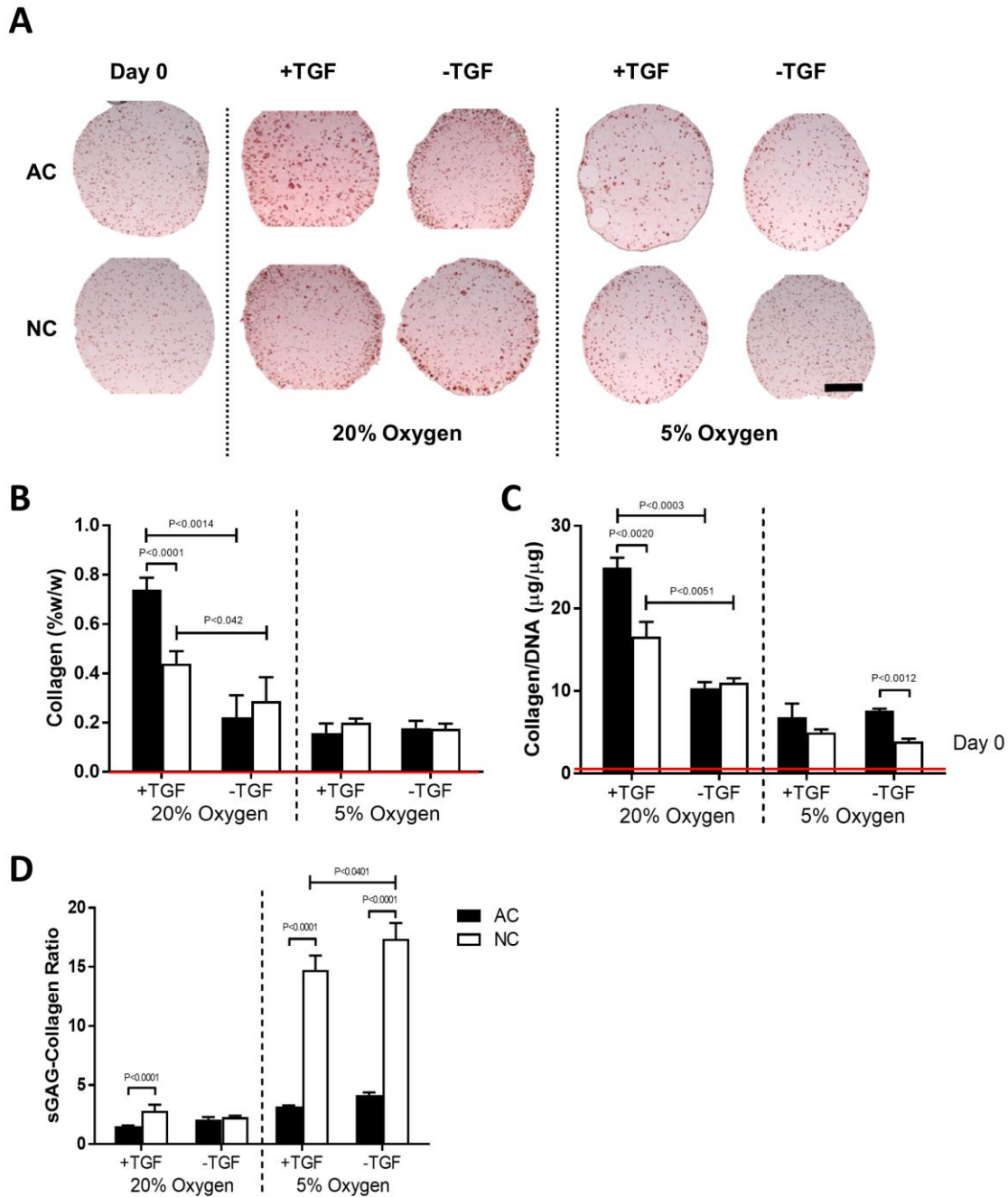


Figure 4

FIG. 4. Collagen accumulation for Articular Chondrocytes (AC) and Nasal Chondrocytes (NC) after 21 days in alginate bead culture subjected to either 20% (normoxic) or 5% (physioxic) oxygen concentration with or without TGF- β supplementation. (A) Histological evaluation with picro-sirius red to identify collagen at day 0 and day 21. Red staining indicates collagen

deposits. Scale bar: 1 mm (B) Collagen content normalised to percentage wet weight (% w/w) (C) Collagen normalized on a per cell basis (Collagen/DNA) and (D) sGAG-Collagen ratio.

Red line indicates baseline values at Day 0.

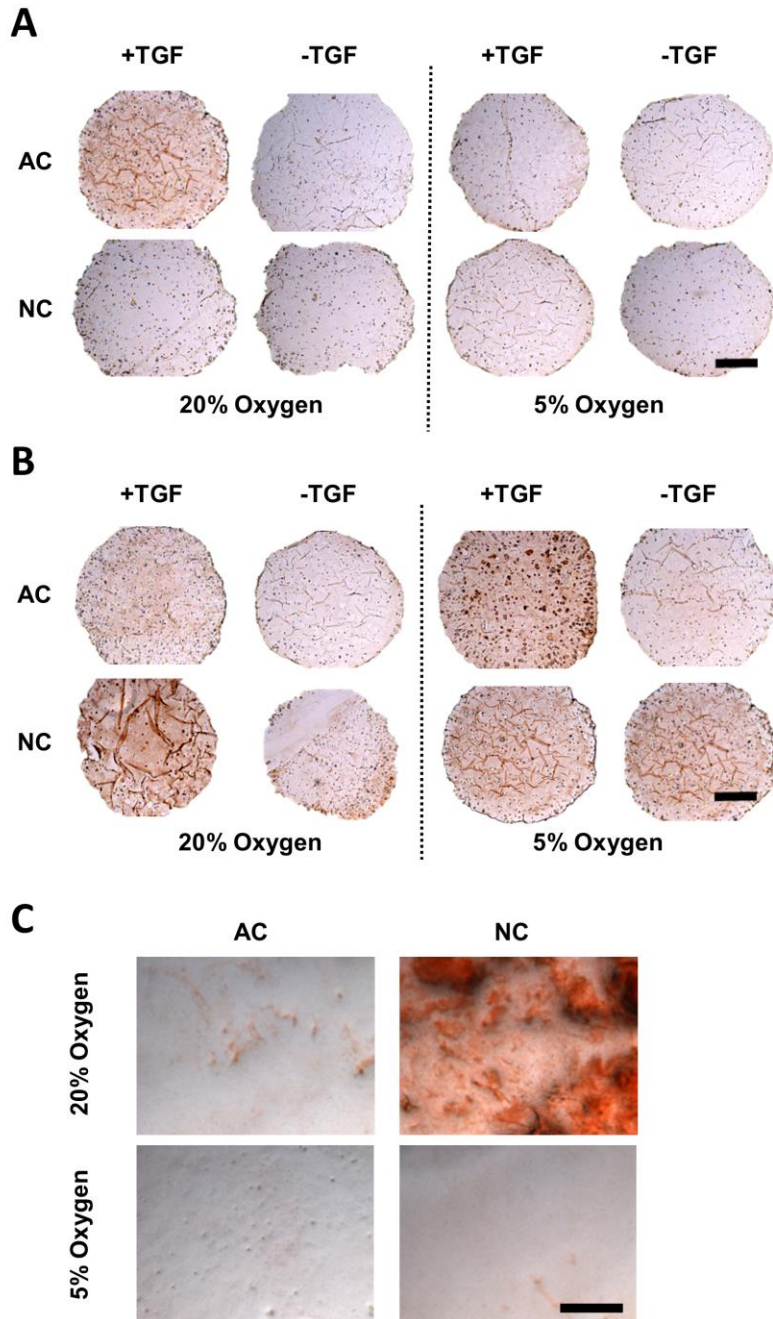
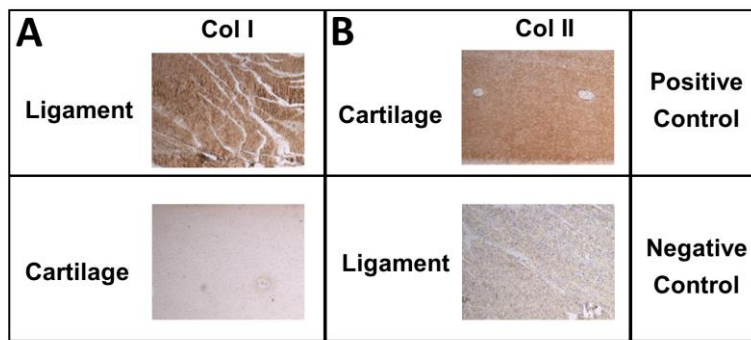


Figure 5

FIG. 5. Immunohistochemical evaluation and in vitro 2D evaluation of osteogenesis. (A) collagen type I and (B) collagen type II for Articular Chondrocytes (AC) and Nasal Chondrocytes (NC) after 21 days in alginate bead culture subjected to either 20% (normoxic) or 5% (physioxenic) oxygen concentration with or without TGF- β supplementation. Scale bar: 1 mm. (C) Alizarin red staining of 2D monolayers cultured at 20% and 5% oxygen concentration after 7 days. Orange–red staining indicate calcium deposits. Scale bar: 1 mm



Supplementary Figure 1

Supplementary FIG. 1. Positive and negative tissue (ligament and cartilage) controls for immunohistochemical assessment for collagen (A) type I, (B) type II.

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